PATENT
ATTORNEY DOCKET 28385/35415

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Application of:	)	For: Anti-Viral Uses of Leflunomide Products
Williams *et al.*	)	
	)	Group Art Unit: 1617
Serial No: 09/529,053	)	
Filed: April 6, 2000	)	Examiner: S. Wang

APPELLANTS' REPLY BRIEF UNDER 37 C.F.R. §41.41

This reply brief is filed pursuant to 37 C.F.R. § 41.41 in response to Examiner's Answer mailed March 24, 2008. The brief is timely filed inasmuch as the two month deadline for filing the reply brief fell on a Saturday of Memorial Day weekend and the next business day was Tuesday, May 27, 2008. The Commissioner is authorized to charge any requisite fees that should be submitted herewith to Deposit Account No. 13-2855 under reference no. 28385/35415.

I. STATUS OF CLAIMS

Claims canceled:	claims 1-33 and 43-44
Claims pending:	claims 34-42, 45 and 46
Claims withdrawn:	none
Claims rejected:	claims 34-42, 45 and 46
Claims on appeal:	claims 34-42, 45 and 46

II. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Rejection of claim 46 under 35 U.S.C. §112, first paragraph, for new matter. There are no art rejections for claim 46.

B. Rejection of claims 34-42 and 45 under 35 U.S.C. §103 for obviousness.

III. ARGUMENT

35 U.S.C. §103 Obviousness Rejection for Claims 34-42 and 45

The following reply is made in response to two issues raised in the Examiner's answer.

(A) Evidence already of record refutes the Examiner's assertion that the unexpected results are not commensurate in scope with the claims because the application only shows the benefit of uridine. Appellants previously supplied evidence showing that (1) other pyrimidine compounds, such as orotic acid and triacetyluridine, are capable of supplying uridine, and (2) other pyrimidine compounds, such as orotic acid, reduce the toxicity of leflunomide administration *in vivo*. Thus, the Examiner is incorrect in suggesting that Appellants' evidence is limited to uridine.

(B) The Examiner failed to provide logical scientific reasoning to support his assertion that any compound containing a pyrimidine moiety will enhance serum levels of uridine, cytidine or thymidine. In particular, his unsupported assertion that anti-viral nucleoside analogs will act to enhance serum levels of the naturally occurring pyrimidine nucleotides uridine, cytidine or thymidine, is completely contrary to the mechanism of action of these drugs. The basis for their anti-viral activity is their ability to act *unlike* natural nucleosides or nucleotides and thereby inhibit an enzyme responsible for DNA synthesis. Therefore the activity of the anti-viral nucleoside analogs is the opposite of providing naturally occurring pyrimidine nucleotides. The Examiner cannot use this defective scientific reasoning to shift the burden to Appellants to prove that nucleoside analogs do not enhance serum levels of uridine, cytidine or thymidine.

A. The Evidence of Unexpected Results is Not Limited to Uridine and is Commensurate in Scope with the Claims

The Examiner persists in rejecting Appellants' evidence of unexpected results to refute the 35 U.S.C. §103 obviousness rejection for claims 34-42 and 45. The Examiner asserts that "The claims read on any pyrimidine compounds, while the application merely shows the benefit of uridine (example 2), not all of its derivatives as herein claimed.

Therefore, the claims are not commensurate in scope with the evidence on the record." (See Examiner's Answer mailed March 24, 2008 paragraph 13).

An inspection of the record will reveal that the Examiner is mistaken. Appellants have, in fact, previously submitted evidence that other pyrimidine compounds such as uridine derivatives can raise uridine levels and reduce the toxicity of leflunomide administration *in vivo*.

1. Triacetyluridine raises uridine levels

Appellants previously provided evidence that another pyrimidine compound, triacetyluridine, can supply uridine and thereby enhance serum levels of uridine. In Appellant's October 12, 2007 Response to the Office Action mailed July 26, 2007 at page 4 paragraph 3, Appellants stated:

> See, e.g., Ashour et al., *Biochemical Pharmacol.*, 51;12:1601-1611 (1996), supplied herewith. *The 2', 3', 5'-tri-O-acetyluridine (TAU) prodrug referenced in Ashour et al. has been shown in human clinical trials to deliver uridine.* See, e.g., Kelsen et al., *J. Clin. Oncol.*, 15:1511-1517 (1997) (Figure 1 of Hidalgo et al., *J. Clin. Oncol.* 18:167-177 (2000) shows that PN401 is 2', 3', 5'-tri-O-acetyluridine), also supplied herewith. [Emphasis added.]

A copy of Ashour et al., *Biochemical Pharmacol.*, 51;12:1601-1611 (1996) is supplied herewith [Appendix B-51]. A copy of Kelsen et al., *J. Clin. Oncol.*, 15:1511-1517 (1997) is supplied herewith [Appendix B-72]. A copy of Hidalgo et al., *J. Clin. Oncol.* 18:167-177 (2000) is supplied herewith [Appendix B-62].

2. Orotic acid raises uridine levels

Appellants previously provided evidence that another pyrimidine compound, orotic acid, also reduces the toxic side effects of leflunomide *in vivo*. See Appellants' December 7, 2006 Response to the Final Office Action mailed November 16, 2006 at page 9 paragraph 3.

A copy of Williams, WO 2006/014827, was supplied with Appellants' Brief [Appendix B-26]. Examples 2 and 3 of this document show that co-administration of orotic acid with leflunomide reduced anemia and increased weight gain.

3. Other evidence confirming unexpected results

Appellants also previously provided evidence confirming that uridine unexpectedly reduces the toxic side effects of leflunomide *in vivo*. See Appellant's December 7, 2006 Response to the Final Office Action mailed November 16, 2006, at page 9 paragraph 2.

A copy of Chong et al., *Transplantation*, 1999 Jul 15;68(1):100-9, was supplied with Appellants' Brief [Appendix B-4]. The abstract of this document states that:

> Toxicities associated with high-does leflunomide (35 mg/kg/day) were anemia, diarrhea, and pathological changes in the small bowel and liver. *These toxicities were significantly reduced by uridine co-administration.* [Emphasis added.]

4. The evidence is commensurate with the claims

The evidence shows that the unexpected benefits, reduction of leflunomide toxicity, arise from the restoration of normal levels of naturally occurring pyrimidine nucleotides, and thus the unexpected benefits are commensurate in scope with the claims, which recite administration of a pyrimidine compound that increases levels of uridine, cytidine or thymidine. Uridine, cytidine and thymidine are the naturally occurring pyrimidine nucleotides that are used as "building blocks" for DNA or RNA, see paragraph 8 of Atwood Declaration [Appendix B-2].

The toxicity of leflunomide is due to inhibition of pyrimidine nucleotide synthesis, which leads to an inadequate level of the pyrimidine nucleotides needed to supply rapidly proliferating cells. However, as shown in the examples, Applicants discovered that inhibition of pyrimidine nucleotide synthesis was not necessary for anti-viral activity, and that restoring normal pyrimidine nucleotide levels with pyrimidine compounds did not interfere with the anti-viral activity of leflunomide product. In fact, utilizing the claimed methods safely permits a higher dose of leflunomide product to be administered, with correspondingly greater anti-viral effect.

The record clearly shows that Appellant has provided evidence of unexpected results not only for uridine (Example 2) but also for other pyrimidine compounds such as orotic acid

and triacetyluridine. Therefore, the Examiner's continued dismissal of Appellant's evidence regarding unexpected results to refute 35 U.S.C. §103 obviousness rejections is improper.

B. Defective Scientific Reasoning Cannot Be Used to Shift the Burden to Appellants

To support the 35 U.S.C. §103 obviousness rejection for claims 34-42 and 45, the Examiner asserts that "All pyrimidine compounds on the record have a pyrimidine moiety, and would have been reasonably expected to add an intermediate in pathways for supplying pyrimidine nucleotide." Therefore, the Examiner concludes, "The burden [is] on appellants to produce evidence showing the pyrimidine compounds cited in the references actually do not possessing [sic] the property as claimed." (See Examiner's Answer 3/24/08 paragraph 12).

The Examiner provided no evidence or scientific reasoning to support his assertion that any compound comprising a pyrimidine moiety would have been reasonably expected to supply uridine, cytidine or thymidine. The Examiner also provided no evidence or scientific reasoning that would support an assertion that the anti-viral nucleoside analogs disclosed in the cited references meet the express limitation in the claims that the pyrimidine compounds enhance serum levels of uridine, cytidine or thymidine. In fact, Appellants' evidence of record shows that the Examiner's assertions are defective and not rational.

The Examiner cited *In re Fitzgerald*, 619 F.2d 67, 70 (CCPA 1980) in support of his conclusion that the burden should be shifted to Appellants to show that the anti-viral nucleoside analogs do not enhance serum levels of these natural pyrimidine nucleotides. However, the Examiner's reliance on *Fitzgerald* and its predecessor cases, *In re Best*, 562 F.2d 1252, 1255 (CCPA 1977) and *In re Swinehart*, 439 F.2d 210, 213 (CCPA 1971) is misplaced under these circumstances. See *Ex parte Jurg Zimmerman*, 2003 WL 25277881 (BPAI 2003) which states:

> We are mindful that there is a line of cases represented by *In re Swinehart*, 439 F.2d 210, 169 USPQ 226 (CCPA 1971) which indicates that where an examiner has reason to believe that a functional limitation asserted to be critical for establishing novelty in the claimed subject matter may, in fact, be an inherent characteristic of the prior art, the examiner possesses the authority to require an applicant to prove that the subject matter shown to be in the prior art does not possess the characteristic relied on. ***Nevertheless, before an applicant can be put to this burdensome task, the examiner must provide***

> ***some evidence or scientific reasoning to establish the reasonableness of the examiner's belief that the functional limitation is an inherent characteristic of the prior art.*** In the case before us, no such evidence or reasoning has been set forward. Id. at 1789.
>
> *Ex parte Jurg Zimmerman* at *4 quoting *Ex parte Skinner*, 2 USPQ2d 1788 (BPAI 1986) [Emphasis added].

In this case, the Examiner has provided no factual evidence or scientific reasoning to establish the reasonableness of his belief that the non-naturally occurring nucleoside analogs disclosed in the prior art could enhance serum levels of uridine, cytidine, or thymidine. On the contrary, Appellants have provided evidence and scientific reasoning that supports the ***opposite*** conclusion, i.e., that the reduction in toxicity with the claimed method occurs because the supply of naturally occurring pyrimidine nucleotides is increased, an effect that would not be expected from administering non-naturally occurring nucleoside analogs.

Appellants provided expert evidence that uridine, cytidine and thymidine are naturally occurring "building blocks" for DNA or RNA, and that compounds that supply these nucleosides would not be expected to have an anti-viral effect. See paragraph 8 of the Atwood Declaration [Appendix B-2]. Moreover, Appellants provided expert evidence that "the definition of pyrimidine compound [as a compound that increases naturally occurring nucleotide levels] ***excludes*** pyrimidine compounds with anti-viral activity." See paragraph 10 of the Atwood Declaration [Appendix B-2]; emphasis added.

The nucleoside analogs disclosed in the cited references exert their anti-viral effect ***precisely because they are non-natural analogs***. Anti-viral nucleoside analogs inhibit the activity of reverse transcriptase, thereby inhibiting viral DNA synthesis. Thus, the basis for their anti-retroviral activity is their ability to act ***unlike*** natural nucleosides. The activity of the anti-viral nucleoside analogs is therefore the opposite of providing naturally occurring pyrimidine nucleotides. These analogs ***do not enhance serum levels of uridine, cytidine or thymidine*** and actually teach away from treating viral infection with a pyrimidine compound that supplies naturally occurring nucleotides such as uridine, cytidine or thymidine.

Thus, the only evidence of record, i.e., that pyrimidine compounds that enhance serum levels of uridine, cytidine and thymidine ***exclude*** pyrimidine compounds with anti-viral activity, supports Appellants' position and contradicts the Examiner's position that the nucleoside analogs in the cited references supply uridine, cytidine or thymidine. Because the Examiner has not met his requisite burden under *Zimmerman* and *Skinner* for invoking

Fitzgerald, it was improper for the Examiner to shift the burden to Appellants to show that the pyrimidine compounds cited in the references actually do not enhance serum levels of uridine, cytidine or thymidine.

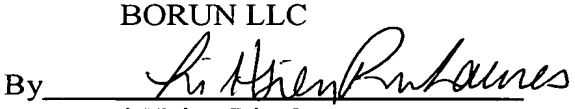
Thus, the cited art's disclosure of nucleoside analogs teaches nothing with respect to administering pyrimidine compounds that enhance serum levels of uridine, cytidine or thymidine to a person suffering from viral infection according to claim 34. Moreover, the pyrimidine compounds recited in the claim are performing a toxicity-reducing function that is unexpected and entirely different from the anti-viral function assertedly disclosed by the cited art. These results thus represent an improvement that is "more than the predictable use of prior art elements according to their established functions." *KSR*, 127 S. Ct. at 1740. For these reasons, among others explained in detail in the present reply brief and Appellants appeal brief, the obviousness rejection should be reversed.

C. Conclusion

The Examiner's legal and factual errors thus necessitate reversal of all obviousness rejections and return of this case to the Examiner for appropriate allowance of the claims.

Respectfully submitted,

MARSHALL, GERSTEIN & BORUN LLC

By 

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APPENDIX B

SUPPLEMENTAL EVIDENCE

Table of Contents for Appeal Brief

Page	Description	When filed, cited and/or entered
B-2	Declaration of Walter Atwood, Ph.D.	Filed by Appellants December 21, 2006 and acknowledged by the Examiner in the non-final Office action mailed on July 26, 2007
B-4	Chong et al., Transplantation, 1999 Jul 15;68(1):100-9	Exhibit D to Appellants' response filed February 27, 2006, and acknowledged as considered in the office action mailed May 23, 2006
B-14	Sommadossi et al., *Antimicrob. Agents Chemother.* 32(7): 997-1001 (1988)	Exhibit B to Appellants' response filed February 27, 2006, and acknowledged as considered in the office action mailed May 23, 2006
B-19	Walker et al., *Antivir. Ther.* 10 suppl. 2:M117-23 (2005) (abstract)	Exhibit C to Appellants' response filed February 27, 2006, and acknowledged as considered in the office action mailed May 23, 2006
B-26	Williams et al., WO 2006/014827	Exhibit E to Appellants' response filed February 27, 2006, and acknowledged as considered in the office action mailed May 23, 2006

Supplemental Table of Contents for Reply Brief

Page	Description	When filed, cited and/or entered
B-51	Ashour et al., *Biochemical Pharmacol.*, 51;12:1601-1611 (1996)	Exhibit E1 to Appellant's Response filed October 12, 2007
B-62	Hidalgo et al., *J. Clin. Oncol.* 18:167-177 (2000)	Exhibit E2 to Appellant's Response filed October 12, 2007
B-72	Kelsen et al., *J. Clin. Oncol.*, 15:1511-1517 (1997)	Exhibit E3 to Appellant's Response filed October 12, 2007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Williams *et al.*	)	
Serial No. 09/529,053	)	Group Art Unit: 1617
Filed: April 6, 2000	)	Examiner: S. Wang
For: ANTI-VIRAL USES OF LEFLUNOMIDE PRODUCTS	)	

DECLARATION OF WALTER ATWOOD, Ph.D.

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

I, Walter Atwood, Ph.D., hereby declare as follows:

1. I am currently a Professor of Medical Science at Brown University. I received a B.S. in Microbiology from the University of Massachusetts at Amherst in 1986 and a Ph.D. in Neurovirology from the University of Massachusetts at Amherst in 1991. I am the author or co-author of numerous peer-reviewed journal articles and book chapters in the field of virology.

2. I have reviewed the text of U.S. patent application no. 09/529,053, attached hereto as Exhibit 1.

3. I have reviewed a copy of claim 34 attached hereto as Exhibit 2, which relates to a method of treating viral infection by co-administering a leflunomide product and a pyrimidine compound without antiviral activity.

4. I understand that the Examiner has objected to the recitation of a pyrimidine compound "without antiviral activity" in claim 34 because he believes that the application does not describe the use of pyrimidine compounds without antiviral activity. I make these statements to address the Examiner's objection.

5. My experience and education permit me to be familiar with what one of ordinary skill in the art would have understood upon reading the application at its March 11, 1998 priority date.

6. The application describes the anti-viral effects of leflunomide product and further describes methods of treating viral infection with leflunomide product. The application also states at page 14, lines 1-6 that:

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> According to another aspect of the invention, a leflunomide product is co-administered with a pyrimidine, such as uridine, in order to reduce its toxicity while maintaining its therapeutic effectiveness. It is contemplated that co-administration with a pyrimidine may allow administration of an anti-viral therapeutically effective amount of leflunomide product with reduced immunosuppressive or toxic side effects.

7. One of ordinary skill in the art as of March 11, 1998 would have understood, from reading the language quoted in paragraph 6, that the inventor(s) contemplated administration of a pyrimidine compound ***to reduce the toxicity of the leflunomide product, not for any anti-viral effect.*** In other words, it is the leflunomide product, not the pyrimidine compound, that would have anti-viral activity.

8. The definition of pyrimidine compound confirms that the contemplated pyrimidine compounds ***would not have anti-viral activity.*** A pyrimidine compound is defined at page 20, lines 12-14 of the application as "compounds useful either directly or as intermediates in pathways for supplying pyrimidine nucleotides (uridine, cytidine and thymidine)." Uridine, cytidine and thymidine are naturally occurring nucleosides, which are used as a "building block" for DNA or RNA, and which have no anti-viral effect. Thus, compounds that supply these nucleosides would not be expected to have an anti-viral effect.

9. The fact that uridine, an exemplary pyrimidine compound, has no anti-viral activity is confirmed in Example 2, Figure 2, which shows that uridine [Ur] alone has no effect on the production of infectious virus. In contrast, the leflunomide product A771726 alone [A77], or A771726 plus uridine [A77+Ur], inhibited infectious virus production.

10. It is clear, from reading the application, that the pyrimidine compounds to be co-administered with leflunomide product were not intended to have antiviral activity. I base my conclusion on the facts that (a) the stated purpose of the pyrimidine compound was to reduce toxicity of the leflunomide product, not for an anti-viral effect, and (b) the definition of pyrimidine compound excludes pyrimidine compounds with anti-viral activity.

11. Therefore, one of ordinary skill in the art as of March 11, 1998, upon reading the application, would have understood that the inventors were claiming the administration of pyrimidine compounds ***without antiviral activity.***

12. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. § 1001), and may jeopardize the validity of the application or any patent issuing thereon.

12/19/06
Date

Dr. Walter Atwood

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TRANSPLANTATION

Vol. 68, 100-109, No. 1, July 15, 1999
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IN VIVO ACTIVITY OF LEFLUNOMIDE

PHARMACOKINETIC ANALYSES AND MECHANISM OF IMMUNOSUPPRESSION

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Background. **Leflunomide is an experimental drug with demonstrated ability to prevent and reverse acute allograft and xenograft rejection. The two biochemical activities reported for the active metabolite of leflunomide, A77 1726, are inhibition of tyrosine phosphorylation and inhibition of dihydroorotate dehydrogenase, an enzyme necessary for *de novo* pyrimidine synthesis. These activities can be distinctly separated *in vitro* by the use of uridine, which reverses the anti-proliferative effects of A77 1726 caused by inhibition of *de novo* pyrimidine synthesis. We report the effect of uridine on the *in vivo* immunosuppressive activities of leflunomide.**

Methods. **We first quantified the serum levels of A77 1726, the active metabolite of leflunomide, after a single treatment of leflunomide (5, 15, and 35 mg/kg). Additionally, we quantified the levels of serum uridine and of nucleotide triphosphates in the liver, spleen, and lymph nodes of Lewis rats after the administration of a single dose of uridine (500 mg/kg; i.p.). Lewis rats heterotopically transplanted with brown Norway or Golden Syrian hamster hearts were treated for 50 or 75 days with leflunomide (5, 15, and 35 mg/kg/day; gavage) alone or in combination with uridine (500 mg/**

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kg/day; i.p.). Hematocrits were determined and the levels of alloreactive or xenoreactive immunoglobulin (Ig)M and IgG were determined by flow cytometric analysis. The allograft and xenografts, small bowel, liver, kidney, and spleen were subjected to pathological examination.

Results. **A linear relationship was observed between the serum A77 1726 concentrations in Lewis rats and the dose of leflunomide administered. Peak A77 1726 concentrations were 20.9, 71.8 and 129.3 mg/l (77.5, 266.1 and 478.8 μM) for the 5, 15, and 35 mg/kg doses of leflunomide, respectively. The concentration of uridine in the serum of normal Lewis rats is 6.5 μM; after i.p. administration of 500 mg/kg uridine, the serum uridine concentrations peaked at 384.1 μM in 15–30 min. The rapid elimination of uridine was not reflected in the lymphoid compartments, and the pharmacokinetics of pyrimidine nucleotides in the spleen resembled that of A77 1726. This dose of uridine, when administered daily (500 mg/kg/day, i.p.), weakly antagonized the immunosuppressive activities of leflunomide (5, 15, and 35 mg/kg/day) in the allotransplantation model. In contrast, in the xenotransplantation model, the same concentration of uridine completely antagonized the immunosuppressive activities of low-dose leflunomide (15 mg/kg/day) and partially antagonized the immunosuppressive activities of high-dose leflunomide (35 mg/kg/day). Toxicities associated with high-dose leflunomide (35 mg/kg/day) were anemia, diarrhea, and pathological changes in the small bowel and liver. These toxicities were significantly reduced by uridine co-administration.**

Conclusion. **These studies reveal that the blood levels of A77 1726 in Lewis rats satisfy *in vitro* requirements for both inhibition of *de novo* pyrimidine synthesis and protein tyrosine kinase activity. Our data also illustrate that the *in vivo* mechanism of immunosuppression by leflunomide is complex and is affected by at least the following four factors: type and vigor of the immune response, availability of uridine for salvage by proliferating lymphocytes, species being investigated, and concentration of serum A77 1726.**

Leflunomide [N-(trifluoromethylphenyl)-methylisoxazol-4-carboxamide; HWA 486 or SU101] is an experimental immunosuppressive drug with demonstrated ability to prevent and reverse acute allograft and xenograft rejection (reviewed in (*1*)). Leflunomide is rapidly converted *in vivo* to the active metabolite, A77 1726. The two biochemical activities ascribed to A77 1726 are inhibition of protein tyrosine kinases and of dihyroorotate dehydrogenase (DHO-DHase*), a key enzyme in the *de novo* synthesis of pyrimidine nucleotides (*2–6*). The significantly lower IC_{50} required *in vitro* to inhibit DHO-DHase relative to tyrosine kinases has led many researchers in this field to suggest that the *in vivo* antiproliferative and immunosuppressive activities of leflunomide, and its active metabolite, A77 1726, result from the inhibition of the enzymatic activity of DHO-DHase (*1*).

Several lines of evidence suggest that this hypothesis may not be supported *in vivo*. First, the serum uridine in human and rodents (5–15 μM (*7, 8*)) could be converted to pyrimidine nucleotides by the salvage pathway, resulting in a normalization of intracellular pyrimidine nucleotide levels, despite inhibition of *de novo* pyrimidine synthesis. Second, when A77 1726 is used *in vitro* at concentrations >50 μM, the antiproliferative activity on T cells and B cells could no longer be completely reversed by the addition of exogenous uridine, suggesting a second mode of activity at these concentrations that is independent of pyrimidine depletion (*9, 10*). Third, patients with a genetic defect in *de novo* pyrimidine synthesis, hereditary orotic aciduria, do not have undue susceptibility to infection, indicating that they are not significantly immunosuppressed (reviewed in (*11*)). *In vitro* cellular immune defects have been reported in some of these patients; however, these immune defects can be attributed to lymphopenia arising from a generalized defect in hematopoiesis, rather than to an intrinsic inability of T cells to proliferate in response to antigen stimulation (*12, 13*).

These observations prompted us to examine whether the immunosuppressive activities of leflunomide in rats and mice are mediated by the inhibition of *de novo* pyrimidine synthesis. *In vitro* studies suggest that the effects of A77 1726 that are caused by inhibition of *de novo* pyrimidine synthesis can be reversed with uridine. Using a similar approach, we report the effects of uridine on the *in vivo* immunosuppressive activities and toxic side effects of leflunomide in mice and rats.

MATERIALS AND METHODS

Extraction and quantitation of A77 1726 from serum. For single-dose pharmacokinetics, Lewis rats not receiving transplants were treated with a single dose of 5, 15, or 35 mg/kg/day leflunomide by gavage. Serum was collected at the indicated times and stored at 70°C before extraction. Serum, 50 μl, was mixed with 25 μl 4'-(trifluoromethyloxy)-acetanilide (TFMO; Sigma, St. Louis, MO), 200 μl 0.5 M HCl, and 4 ml extraction solvent (1:1 pentane and dichloromethane; Sigma). The mixture was vortexed for 1 hr, then centrifuged at 2000 g for 5 min (Centra-8, IEC, Needham Heights, MA). After freezing the mixture for 1 hr at −20°C, the organic phase was decanted into a new tube and dried under a hood at room temperature. Then 200 μl of reconstituting solution (1:1 acetonitrile and water) was added, and the mixture was vortexed and centrifuged at 2000 g for 2 min. Finally, 65 μl of the mixture was analyzed by high-performance liquid chromatography (HPLC) (Waters, Milford, MA), using a Symmetry C18 column (4.6 × 250 mm; Waters). A77 1726 was separated with a mobile phase composed of 50% acetonitrile and 50% buffer (25 mM KH_2PO_4). The corresponding peak of A77 1726 was compared with a standard of purified A77 1726 (a gift from Robert R. Bartlett, Hoechst Marion Roussel, Wiesbaden, Germany), and the concentrations were calculated on the basis of a standard curve of purified A77 1726.

Extraction and quantitation of uridine from serum. For single-dose pharmacokinetics, Lewis rats not receiving transplants were treated with a single dose of uridine. Serum was collected at the indicated times and stored at 70°C before extraction. Serum samples were diluted two fold in 0.9% NaCl, and uridine was extracted by the addition of an equal volume of 0.8 M trichloric acid and then neutralized with an equal volume of 0.5 M tri-n-octylamine in Freon. Serum uridine was detected by HPLC, using a Lichrosorb-10RP-18 column (Whatman, Alltech, Deerfield, IL) and an elution solution (5 mM KH_2PO_4, pH 3.8), at a flow of 1 ml/min. The uridine peak was identified by its retention time and spectrum compared with a uridine standard (Sigma). Uridine concentrations were calculated on the basis of a standard curve.

Extraction and quantitation of nucleotide triphosphate from tissues. For single-dose pharmacokinetic analysis, normal Lewis rats were treated with a single dose of uridine. The spleen, lymph nodes,

* Abbreviations used: DHO-DHase, dihydroorotate dehydrogenase; Ig, immunoglobulin; HPLC, high-performance liquid chromatography; Ka, rate of absorption; Ke, rate of excretion; PCV, packed cell volume.

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and liver (100 mg tissue) were homogenized and nucleotide triphosphates were extracted with 0.4 M trichloric acid and neutralized with an equal volume of 0.5 M tri-n-octylamine in Freon 113, as previously described (*9, 14*). Nucleotides were separated using a Whatman anion exchange column (Particil 10 SAX, Alltech) and a linear gradient elution of potassium phosphate buffer, pH 4.5 (10–500 mM). The corresponding peaks of four nucleotides were detected by HPLC (Waters), and the concentrations were calculated on the basis of a standard curve of purified nucleotides (Sigma).

Pharmacokinetic and statistical analysis. The pharmacokinetic analysis of serum A77 1726, uridine, and tissue UTP levels were conducted using a nonlinear regression analysis with a Gaussian algorithm. The time-concentration data were fitted to the open, one-compartment, extravascular model:

$$C_t = C^\circ(\exp(-K_e{}^*t) - \exp(-K_a{}^*t))$$

where C_t, C°, K_e, K_a, and t are the serum concentration at time t, the theoretical initial concentration, the excretion constant, the absorption constant, and the time after drug administration. The best fit values for C°, K_e, and K_a were used to calculate the terminal half-life ($T_{1/2}$) using the formula: $T_{1/2} = 0.693/K_e$. The area under the curve was calculated using the trapezoidal method. Statistical differences between pharmacokinetic parameters were analyzed using a *t* test or analysis of variance.

Transplantation model and drug treatment. Lewis or brown Norway rats, and Golden Syrian hamsters were purchased from Harlan Labs (Indianapolis, IN). Balb/c and C3H mice were purchased from Jackson Labs (Bar Harbour, ME). Heart grafts were heterotopically transplanted into the abdomen of the recipients after a modified protocol described by Ono *et al.* (*15*). Leflunomide (5–35 mg/kg/day, custom synthesized for research purposes) was suspended in 1% carboxymethyl cellulose and administered by gavage. Uridine (Sigma) was dissolved in 0.9% NaCl for daily i.p. injections. The transplanted hearts were monitored daily, and rejection was defined as the complete cessation of pulsations in the transplanted heart.

Packed cell volume. Rats were bled every 2 weeks or on the day they were killed, through the orbital vein, using a microhematocrit capillary tube (Baxter, Deerfield, IL). The blood was centrifuged for 15 mins at 550 g, and the percentage of packed cell volumes was determined with a micro-hematocrit capillary tube reader (Critocaps, Oxford Lab).

Quantification of allo-specific and hamster-specific IgM and IgG titers. Quantification of allo-specific or hamster-specific antibodies was performed, as previously described (*16, 17*). Lymphocytes (5×10^5) from lymph nodes isolated from brown Norway rats or erythrocytes (10^6) from Golden Syrian hamster were incubated with diluted, heat-inactivated test serum or control naive Lewis rat serum (1:20 dilution) for 30 min at 4°C. Lymphocytes were washed with phosphate-buffered saline, and erythrocytes were washed in 4% (weight/volume) sodium citrate/phosphate-buffered saline. The cells were then stained with phycoerythrin-conjugated F(ab')2 anti-rat immunoglobulin (Ig)M or fluorescein isothiocyanate-conjugated F(ab')2 anti-rat IgG (Jackson ImmunoResearch, West Grove, PA). After staining, the erythrocytes and lymphocytes were washed, fixed in 1% formalin, and analyzed using a flow cytometer (Ortho Cytoron Absolute, Ortho Diagnostic Systems, Raritan, NJ).

Histology and immunohistochemistry. Sections of the spleen, liver, kidney, and small bowel were collected, imbedded in frozen tissue matrix CO.C.T. compound (Sakura Finetek U.S.A., Torrance, CA), and snap-frozen in liquid nitrogen. Sections of these tissues, 5 μm, were made and fixed in 10% formalin. These sections were then stained in hematoxylin and eosin solutions. Allografts were scored according to a modified cardiac biopsy grading by Billingham *et al.* (*18*). Grade 0 (no acute rejection) indicates no evidence of acute rejection or myocyte damage; Grade 1A (focal, mild acute rejection) indicates focal, perivascular, or interstitial infiltrate of mononuclear cells with no myocyte damage; Grade 1B (diffuse, mild, acute rejection) indicates a more diffuse, perivascular or interstitial infiltrate of mononuclear cells with no myocyte damage; Grade 2 (focal, moderate acute rejection) indicates a few focal aggressive inflammatory infiltrate with focal myocyte damage; Grade 3A indicates multifocal aggressive inflammatory infiltrate with myocyte damage; Grade 3B indicates diffuse aggressive inflammatory infiltrate with myocyte necrosis; and Grade 4 indicates diffuse aggressive inflammatory infiltrate with myocyte necrosis, hemorrhage, edema, and vasculitis. Sections for immunohistochemical analysis were fixed in cold acetone and stained with monoclonal antibodies against rat IgM, IgG, TCR$\alpha\beta$, and ED1, using a modified ABC method, as previously described (*16, 19*).

RESULTS

Single dose pharmacokinetics of A77 1726. Lewis rats received by gavage three different doses of leflunomide (5, 15, 35 mg/kg). Sera were harvested from 4–6 individual rats at the indicated times, and the active metabolite of leflunomide, A77 1726, in the serum was extracted and quantified by HPLC. Increasing concentrations of leflunomide resulted in a dose-dependent increase in A77 1726 in the serum (Fig. 1A). A linear relationship was observed between the peak concentrations and the area under the curve, and the dose of A77 1726 administered (Fig. 1B). The A77 1726 peak concentrations were 20.9, 71.8, and 129.3 mg/l (77.5, 266.1, and 478.8 μM) for the 5, 15, and 35 mg/kg doses of leflunomide, respectively (Fig. 1A). These peak concentrations were observed at 6–8 hr (Fig. 1A), and the mean terminal half-life ($T_{1/2}$) ranged from 3.5 to 5.0 hr, irrespective of the administered leflunomide dose (Table 1). The excretion constant (Ke) and absorption constant (Ka) were not statistically significant between treatment groups ($P > 0.05$), and the excretion of A77 1726 followed first-order kinetics even at highest dose (Table 1). Similar analyses performed with Balb/c mice given a single, oral dose of leflunomide (35 mg/kg) revealed similar peak A77 1726 concentrations (140 mg/l=514 μM) and Ka, compared with Lewis rats; however, the Ke, was significantly

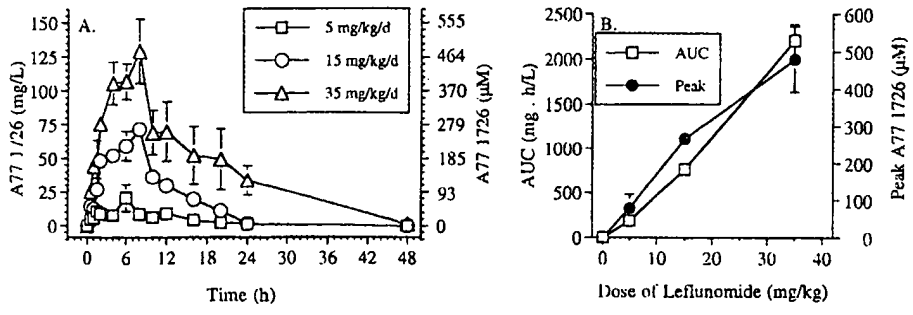

FIGURE 1. (A) Pharmacokinetics of A77 1726 after a single, oral dose of leflunomide in Lewis rats. Serum was harvested at the indicated times, and A77 1726 concentrations determined by HPLC. Data are presented as mean concentrations of 4–6 rats/group, and bars represent SEM. (B) The relationship between the AUC (mg · h/L) and peak concentrations of A77 1726 (μM) and the dose of leflunomide administered.

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TABLE 1. Pharmacokinetic parameters of serum A77 1726 following leflunomide administration

Treatment	Ke (ml · min/kg)	Ka (ml · min/kg)	T1/2 (h)	AUC (mg · h/L)
Single-dose leflunomide treatment in Lewis rats				
5 mg/kg leflunomide	0.258±0.112	0.484±0.138	4.42±0.16	183.0±19.6
15 mg/kg leflunomide	0.190±0.005	0.197±0.004	3.49±0.19	764.5±24.0
35 mg/kg leflunomide	0.150±0.023	0.204±0.046	4.97±0.77	2207.3±173.8
Single-dose leflunomide treatment in Balb/c mice				
35 mg/kg leflunomide	0.073±0.032	0.567±0.132	15.02±4.57	3446.5±973.3

slower ($P<0.05$; Table 1) resulting in a longer terminal half live of serum A77 1726.

Single dose uridine pharmacokinetics. *In vitro* and *in vivo* studies have indicated that uridine can be used to counter the effects resulting from the inhibition of *de novo* pyrimidine synthesis (*2–6*, *14*, *20*). We measured the levels of serum uridine in Lewis rats before and after the administration of a single dose of uridine (500 mg/kg, i.p.). Consistent with previous reports, the mean concentration of uridine in the serum of normal Lewis rats was 6.5±0.9 μM (n=13) (*7*). A single dose of uridine (500 mg/kg) administered i.p. resulted in a rapid increase in the concentrations of serum uridine. Maximum concentrations were observed within 15–30 min and reached a peak concentration of 384.1±53.5 μM. Serum uridine was rapidly cleared and returned to baseline 4 hr after uridine administration (Fig. 2A).

Intracellular nucleotides following a single dose of uridine. We next determined whether the elevated concentrations of serum uridine resulted in increased intracellular pyrimidine nucleotides in the spleen, lymph nodes, and liver. A cohort of 26 Lewis rats were treated with a single dose of uridine (500 mg/kg, i.p.). The rats were killed after 0, 1, 3, 6, 12, and 24 hr (n=4–5 per group), and lymph nodes and approximately 100 mg of liver and spleen tissue harvested. The nucleotides were extracted from the tissues by trichloroacetic acid, and the concentrations of tissue UTP, CTP, ATP and GTP determined by HPLC. The administration of uridine resulted in 4.1-, 3.6-, 2.4-, and 1.5-fold increases in UTP, CTP, ATP, and GTP, respectively, in the spleen (Fig. 2B–D). The tissue nucleotide concentrations remained at these levels for up to 6 hr and gradually declined to baseline 24 hr after uridine administration. The $T_{1/2}$ of tissue UTP in the spleen was 12.72 hr, with a Ka and Ke of 0.216 and 0.05 ml · min/kg, respectively. Statistical analysis indicated that the pharmacokinetics of UTP levels in the spleen after uridine administration was not significantly different ($P>0.05$) from the pharmacokinetics of A77 1726 after the administration of leflunomide.

After uridine administration, the levels of UTP and CTP increased 4.9- and 2.8-fold, respectively, in the liver tissue and 1.7- and 1.3-fold, respectively, in lymph node cells (Fig. 2B–D). The levels of ATP and GTP were also elevated 3.1- and 2.9-fold, respectively, in the liver and 1.5- and 1.3-fold in the lymph node cells after uridine administration (data not shown). We do not have an explanation for the concomitant increase in ATP and CTP levels but speculate that it could reflect uridine-stimulated increases in metabolic activity in these tissues.

Effect of uridine on the ability of leflunomide to control acute allograft rejection in Lewis rats. Brown Norway hearts transplanted into untreated Lewis rats were rejected in 6–8 days. Treatment with 5 or 15 mg/kg/day of leflunomide resulted in the survival of the allografts for >50 days, whereas

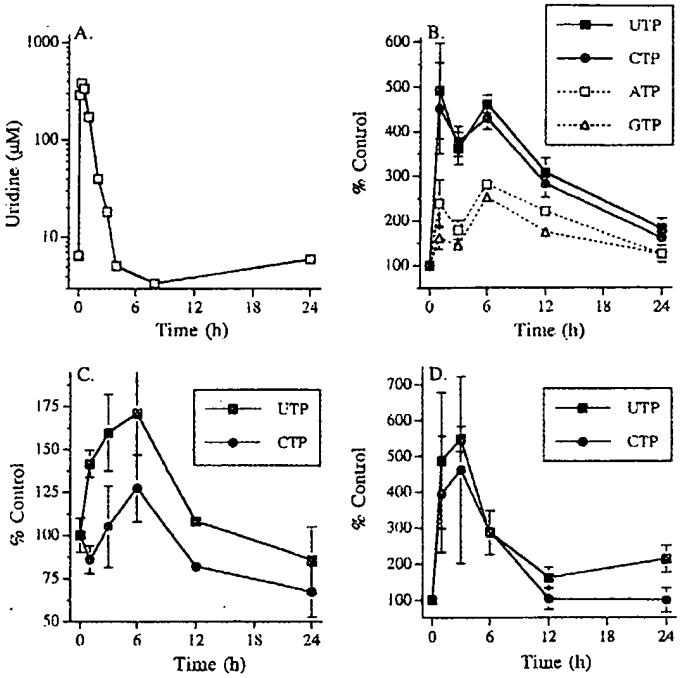

FIGURE 2. Pharmacokinetics of uridine after a single, i.p., dose of uridine (500 mg/kg) in Lewis rats. (A) Serum was harvested at the indicated times and serum uridine concentrations determined by HPLC. Data are presented as mean of 4–13 rats/group, and bars represent SEM. (B–D) Pharmacokinetics of pyrimidine and purine nucleotides in the spleen (B), liver (C) and lymph node (D) after a single, i.p., dose of uridine (500 mg/kg) in Lewis rats. Lewis rats were killed at the indicated times (4–6 rats/group), and the nucleotides extracted following protocols described under Materials and Methods. Data are presented as percentages of untreated controls, and bars represent SEM. The baseline concentrations of UTP, CTP, ATP, and GTP in the liver tissue were 92.0, 4.0, 357.7, 74.8 pg/mg; in the spleen tissue were 28.9, 21.0, 211.8, and 50.9 pg/mg, and in the lymph nodes tissues were 8.8, 7.3, 55.8, and 8.5 pM/20×10^6 cells, respectively.

treatment with 35 mg/kg/day resulted in the sacrifice of all Lewis recipients with beating allografts in <30 days after the transplant because of leflunomide-related toxicities. Uridine (500 mg/kg/day, i.p.) co-administration with leflunomide did not significantly alter allograft survival, and all the hearts were beating on day 50 in the Lewis recipients receiving 5 or 15 mg/kg/day leflunomide plus uridine (Table 2). In the 35 mg/kg/day leflunomide plus uridine group, the toxicity of leflunomide was significantly reduced and 4 of the 5 Lewis recipients were alive, with beating allografts on day 50 after the transplant (Table 2). One of the Lewis recipients in this combination-treatment group died on day 39, with a beating allograft.

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TABLE 2. Effect of uridine on the ability of leflunomide to prevent the rejection of allograft (brown Norway) hearts by Lewis rats[a]

Treatment	Allograft survival (d)	Mean (d)	Histological scores
None	6, 6, 7, 7, 7, 7, 7, 8	6.9±0.2	3A–3B (X8)
Lef (5)	>50 (X8)	>50	2, 2, 2, 2, 3A, 3A, 3A, 3B
Lef (5)+Uridine	>50 (X8)	>50	2, 2, 2, 3A, 3A, 3B, 3B, 3B
Lef (15)	>50 (X6)	>50	1A, 1A, 1A, 1A, 1B, 2
Lef (15)+Uridine	>50 (X6)	>50	1A, 1A, 1B, 2, 2, 2
Lef (35)	>13, >26, >28, >29, >29	>25.0±3.0	0, 0, 0, 1A, 1A
Lef (35)+Uridine	>37, >50 (X4)	>47.4±2.6	0, 1A, 1A, 1A, 1A

[a] Uridine was administered i.p. at 500 mg/kg (once a day), whereas leflunomide was administered orally at 5, 15, or 35 mg/kg daily for 50 days. Survival of leflunomide-treated Lewis rats or grafts were calculated from the day of transplantation, and presented as mean±SE. Histological scores were determined when the rats were killed or at the end of the experiment (day 50 after the transplant).

[b] > indicates that Lewis rats died of leflunomide-induced toxicity with beating allografts, or were killed at the end of the experiment (day 50 after the transplant).

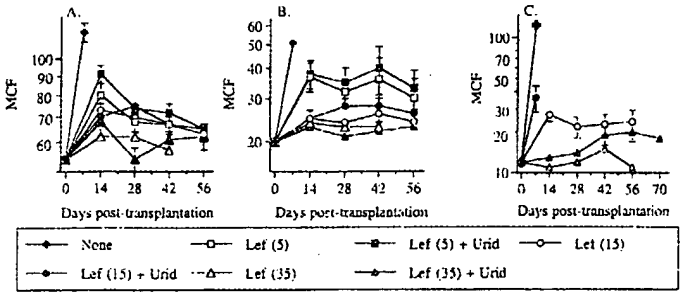

FIGURE 3. Levels of alloreactive IgM (A) and IgG (B) and xenoreactive IgM (C) in Lewis rats treated with leflunomide (Lef 5, 15, or 35 mg/kg/day) alone or in combination with uridine (Urid; 500 mg/kg/day) at the indicated days after the transplant. The relative amounts of alloreactive IgM and IgG and xenoreactive IgM were quantified by flow cytometric analysis, and data are presented as mean channel fluorescence (3 decade log scale)±SE. There were 3–6 rats per group.

We have previously reported that allograft rejection in this model is accompanied by an increase in the titers of alloreactive IgM and IgG (Fig. 3A, B). We here confirm that leflunomide, in a dose-dependent manner, inhibited the increase in both IgM and IgG titers. Co-administration of uridine resulted in a modest increase in the titers of alloreactive IgM and IgG in Lewis rats receiving a 5 mg/kg/day dose of leflunomide but had minimal effects at the higher doses of leflunomide.

On day 50 after the transplant, or at the time the rats were killed, the hearts were harvested and subjected to histological examination. Despite daily treatment with leflunomide (5 mg/kg/day) for 50 days, the allografts demonstrated a mild to moderately intense inflammatory infiltrate and some myocyte necrosis (data not shown). Cellular rejection was significantly reduced in allografts when the recipients were treated with a higher dose of leflunomide (15 mg/kg/day; Fig. 4A, B) and there were no signs of rejection in the allografts harvested from Lewis rats receiving the highest dose of leflunomide (35 mg/kg/day) at the time they were sacrificed (days 13–29 after the transplant; data not shown). In the group receiving uridine (500 mg/kg/day) and leflunomide (5, 15, or 35 mg/kg/day), only slightly exacerbated cellular rejection was observed in the allografts examined on day 50 after the transplant (Table 2, Fig. 4E, F). Some foci of infiltrating T cells and ED1 macrophages were observed, and marginally increased deposition of IgM in the leflunomide plus uridine groups compared to that in the leflunomide (15 mg/kg/day) monotherapy groups (Fig. 4E, F). There was minimal deposition of IgG in the allografts from all leflunomide monotherapy and leflunomide plus uridine groups (data not shown).

Leflunomide induced a dose-dependent reduction in the lymphoid compartments in the spleens of Lewis rats transplanted with Brown Norway hearts. The T lymphocyte zones of the periarteriolar lymphocyte sheath and the B cell zones, located in the follicles, marginal zones, and red pulp (especially around the pulp arterioles) were significantly reduced by leflunomide (15 mg/kg/day; Fig. 4C, D). Uridine completely reversed the effects of leflunomide on the lymphoid compartments in the spleen (Fig. 4G, H), suggesting that, although uridine is able to reverse the antiproliferative effects of leflunomide in the spleen, it has only modest effects on the ability of leflunomide to control allograft rejection.

Effect of uridine on the ability of leflunomide to control acute allograft rejection in C3H mice. Balb/c hearts transplanted into C3H mice were rejected in 8–10 days without immunosuppressive therapy (Table 3). Leflunomide at a dose of 30 mg/kg/day delayed rejection for 21–31 days (31.0±1.8). At this dose, there was no detectable toxicity in the mouse as a result of leflunomide administration. The lack of toxicity probably reflects the reduced sensitivity of mouse DHO-DHase to leflunomide compared with the rat enzyme (*2, 3, 21, 22*). The mean percent of packed cell volume (PCV) in leflunomide-treated C3H mice, measured on the day of rejection, was 52.3±5.7, while in untreated controls was 62.0±2.8. Co-administration of uridine (500 mg/kg/dose; i.p., twice a day) with leflunomide resulted in a slightly more rapid rate of allograft rejection in 19–31 days (23.8±2.7; N=5). This was the maximum dose of uridine that could be administered in this experimental protocol as 4 of 9 mice died of uridine-related toxicity. The mean percent PCV in the surviving recipients, measured on the day of rejection, was 47.2±4.4%.

Effect of uridine on the ability of leflunomide to control acute xenograft rejection in Lewis rats. The rejection of hamster grafts by Lewis rats is mediated by anti-hamster IgM produced in a T-independent and T-dependent manner (*23–25*). We have previously reported that leflunomide at 15 mg/kg/day, but not at the 5 mg/kg/day dose, can prevent acute xenograft rejection in the hamster-to-Lewis transplantation model (*17*). Leflunomide at 15 mg/kg/day resulted in xenograft survival ranging from 48 to ≥75 days (mean graft survival was >63.2±9.7 days). When leflunomide was in-

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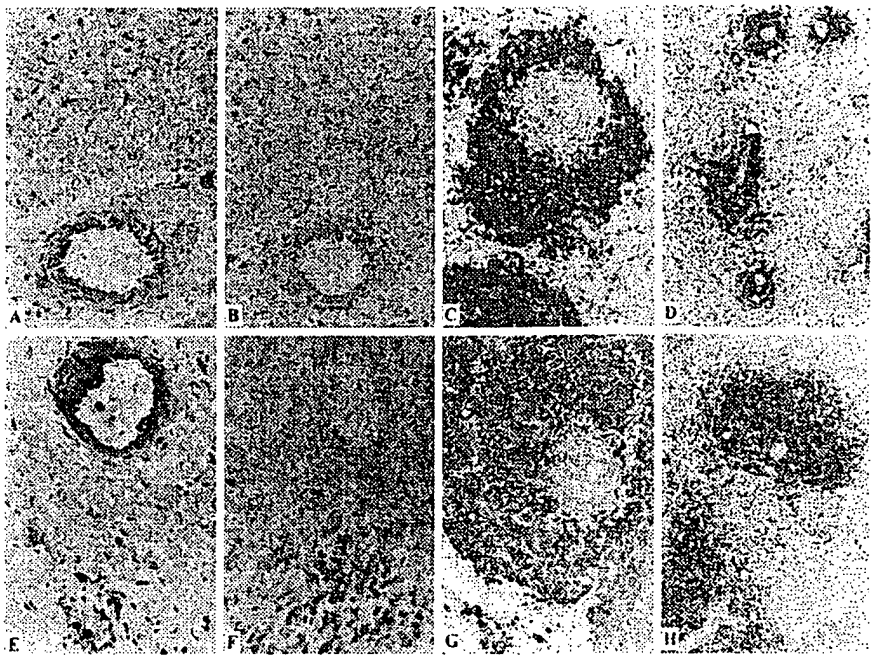

FIGURE 4. The ability of leflunomide (15 mg/kg/day) to inhibit allograft rejection (A and B) was minimally affected by uridine (500 mg/kg/day) co-administration (E and F). The proliferation of T and B cells in the recipient spleen in response to allograft stimulation was inhibited by leflunomide (15 mg/kg/day) (C and D), and this inhibition was reversed by uridine (500 mg/kg/day) co-administration (G and H). A and E: IgM deposition; B and F: TCR$\alpha\beta^+$ T lymphocytes; C and G: B cells (IgM^-) regions in the recipient spleen; D and H: TCR$\alpha\beta^+$ T cell regions in the spleen.

TABLE 3. Effect of uridine on the ability of leflunomide to control allograft (Balb/c) rejection in C3H mice[a]

Treatment	Allograft survival (d)	Means±SE
None	8, 9, 9, 10, 10	9.2±0.8
Uridine	8, 9, 9, 10, 11	9.4±1.1
Lef (30)	21, 27, 29, 30, 31	31.0±1.8
Lef (30)+Uridine	19, 19, 20, 30, 31	23.8±2.7

[a] Uridine was administered i.p. at 1000 mg/kg/day (500 mg/kg/dose given twice a day), whereas leflunomide was administered orally at 30 mg/kg daily from the day of the transplant until rejection. Data are presented as mean±SE. On days 5, 10, 12, and 13 after the transplant, 4 other recipients in this group, with functioning xenografts, died of uridine toxicity. This observation indicated that 1000 mg/kg/day is the maximum tolerable dose of uridine. The rest of the C3H recipients were killed on the day of graft rejection.

creased to 35 mg/kg/day, all the Lewis recipients died of, or were killed because of, leflunomide toxicity before the end of the experiment (75 days). The mean survival of the Lewis recipient was 36.0±14.0 days; however, at the time of death or sacrifice, all xenografted hearts were beating (Table 4).

We next tested the effect of uridine coadministration on the ability of leflunomide to prevent acute rejection in this transplantation model. Uridine 500 mg/kg/day coadministration completely antagonized the immunosuppressive activity of 15 mg/kg/day leflunomide, and the xenografts were rejected in 8.4±0.2 days. In contrast, co-administration of uridine 500 mg/kg/day and 35 mg/kg/day leflunomide resulted in long-term xenograft survival for up to 75 days in 3 of 5 recipients; the remaining 2 recipients were killed because of leflunomide toxicity with beating xenografts (Table 4).

As previously reported, pathological analysis of the xenografts on day 50 after the transplant revealed significant vascular injury, indicative of chronic rejection, when leflunomide was used at a dose of 15 mg/kg/day (*26, 27*). Xenografts removed after 10 days of leflunomide monotherapy (15 mg/kg/day) revealed minimal signs of rejection (Fig. 5A, B). A pathological examination of the grafts from rats treated with leflunomide plus uridine (average survival of 8.4 days) revealed severe acute rejection characterized by intense IgM deposition, arterial necrosis, thrombosis, and myocyte coagulating necrosis with a mild to moderate infiltrate comprising neutrophils and macrophages (Fig. 5E, F). At the time of sacrifice of Lewis rats treated with 35 mg/kg/day leflunomide (mean of 36.0±14.0 days after the transplant), the xenografts seemed histologically normal, with no signs of inflammatory cell infiltration or IgM deposition (Fig. 5C, D). Hearts from Lewis recipients treated with leflunomide (35 mg/kg/day) and uridine revealed mild mononuclear intracellular infiltration and IgM deposition, with minimal myocyte necrosis in 4 of 5 grafts (Fig. 5G, H). One graft had severe mononuclear cell infiltration with arterial intimal thickening, a feature characteristic of chronic rejection (data not shown).

We have previously reported that graft rejection in this concordant xenotransplantation model is accompanied by an increase in the titers of xenoreactive IgM but minimal increases in IgG (Fig. 3B) (*17*). We here confirm that leflunomide significantly inhibited the increase in xenoreactive IgM titers at a dose of 15 mg/kg/day and completely inhibited the increase in the xenoreactive IgM titers at the 35 mg/kg/day dose. Immunohistochemical analysis confirmed that leflunomide was able to inhibit xenoantibody production in a dose-dependent manner, with significant IgM deposition in the xenografts on day 75 after the transplant in groups treated with 15 mg/kg/day leflunomide (data not shown) (*26*), and minimal IgM deposition in the 35 mg/kg/day group (Fig. 5B and 5D).

In the groups in which uridine was co-administered with leflunomide, only marginally higher titers of circulating xenoreactive IgM were observed at the 15 mg/kg/day dose, and uridine had no detectable effect on the ability of the higher leflunomide dose to inhibit xenoantibody production. Immunohistochemical analysis indicated minimal IgM deposition in the xenografts from the leflunomide (15 mg/kg/day) monotherapy group on day 10 after the transplant (Fig. 5B), and dense IgM deposition in the xenograft at the time of rejection

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TABLE 4. Effect of uridine on the ability of leflunomide to control xenograft (hamster hearts) rejection in Lewis rats[a]

Treatment	Xenograft survival (d)	Mean (d)	Histological scores
None	3, 4, 4, 4, 4, 4, 4, 4	3.9±0.3	4 (AR; X9)
Lef (5)	6, 6, 7, 7, 7, 7, 8, 14	7.7±2.5	4 (AR; X9)
Lef (5)+Uridine	ND		ND
Lef (15)	48, 57, 57, 59, 60, 63, 75, >75[b] >75	>63.2±3.2	4 (CR; X9)
Lef (15)+Uridine	8, 8, 8, 9, 9	8.3±0.25	3B, 4, 4, 4 (AR)
Lef (35)	>24, >30, >31, >35, >60	>35.8±6.3	0, 0, 0, 0, 1A (AR)
Lef (35)+Uridine	>34, >34, >75, >75, >75	>58.3±10	1B, 1B, 1B, 2, 3B

[a] Uridine was administered i.p. at 500 mg/kg (once a day) whereas leflunomide was administered orally at 5, 15, or 35 mg/kg daily for up to 75 days. Survival of Lef-treated Lewis rats or grafts were calculated from the day of the transplant and presented as mean±SE. Xenografts were harvested on the day the rats were killed or at the end of experiment (day 75 after the transplant). Histological scores were determined as described under Materials and Methods.

[b] > indicates that the Lewis recipients died or were killed with beating grafts on day 75 after the transplant. AR, acute rejection; CR, chronic vascular rejection.

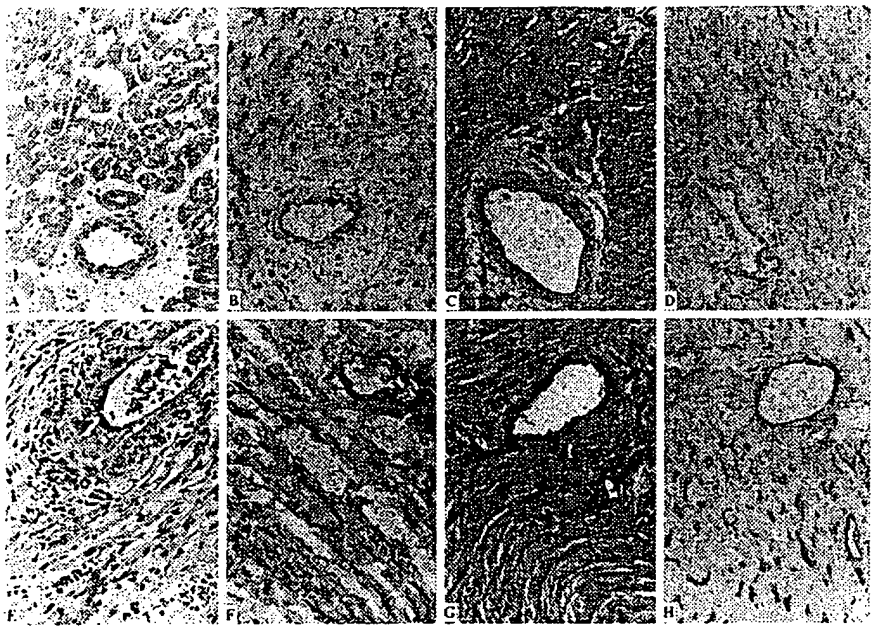

FIGURE 5. The ability of leflunomide (15 mg/kg/day (A, B, E, F) and 35 mg/kg/day) (C, D, G, H) to inhibit xenograft rejection was significantly reversed by uridine co-administration (E–H). Xenografts were harvested on day 10 after transplant (A and B), rejection (D and F), day of sacrifice (C and D), or day 75 after transplant (G and H). A, C, E, and G: HE staining; B, D, F and H: IgM immunostaining.

(day 8–10 after the transplant) in the group treated with 15 mg/kg/day of leflunomide plus uridine (Fig. 5F). There was also marginally more IgM deposition in the xenografts on day 75 in the groups treated with 35 mg/kg/day of leflunomide plus uridine (Fig. 5H), compared with the leflunomide monotherapy group (Fig. 5D). These results indicate that uridine can reduce the ability of leflunomide to control xenoreactive IgM production in the hamster-to-Lewis concordant xenotransplantation model.

Effect of uridine on the toxic side-effects of leflunomide in Lewis rats. Lewis rats with either an allograft or a xenograft, and treated with leflunomide at a dose of 35 mg/kg/day, survived for a mean of 30.5 days (Fig. 6A), with only one of 10 rats surviving <50 days. When the same dose of leflunomide was administered with uridine (500 mg/kg/day), the mean survival of Lewis rats was significantly enhanced, and 7 of 10 Lewis rats were alive after 50 days (Fig. 6A). These observations suggest that this dose of uridine could antagonize the toxic side effects of high-dose leflunomide. Lewis recipients treated with 15 mg/kg/day of leflunomide alone, or in combination with uridine, exhibited minimal signs of toxicity.

Typical signs of leflunomide toxicity in Lewis rats are anemia and diarrhea. We measured the percent of PCV every 14 days after the transplant in the Lewis rats receiving either allografts or xenografts. Treatment with 35 mg/kg/day of leflunomide resulted in a rapid decline in the percentage of PCV (Fig. 6B). In contrast, in the groups receiving uridine and 35 mg/kg/day of leflunomide, the decline in percent of PCV was delayed (Fig. 6B). In the Lewis recipients treated with 15 mg/kg/day leflunomide alone, or combination with uridine, there was no significant drop in the percentage of PCV for the duration of the experiment.

At the end of the experiment (either natural death or being killed), Lewis rats were subjected to a complete autopsy. Histological signs of leflunomide toxicity (35 mg/kg/day) were observed primarily in the small bowel and liver (Fig. 7A, B). Epithelial abnormalities were observed in the intestinal mucosa of the small bowel of rats treated with high-dose leflunomide (35 mg/kg/day). In particular, the villi were short and wide and mature intestinal epithelial cells, including goblet cells, brush border absorption epithelium, and Paneth's cells, were partially or completely substituted by immature low columnar cells, with or without dysplasia (Fig. 7A). These epithelial abnormalities could be caused by atrophy, dedifferentiation, or inhibition of regeneration of the intestinal mucosa by high-dose leflunomide. Uridine significantly pre-

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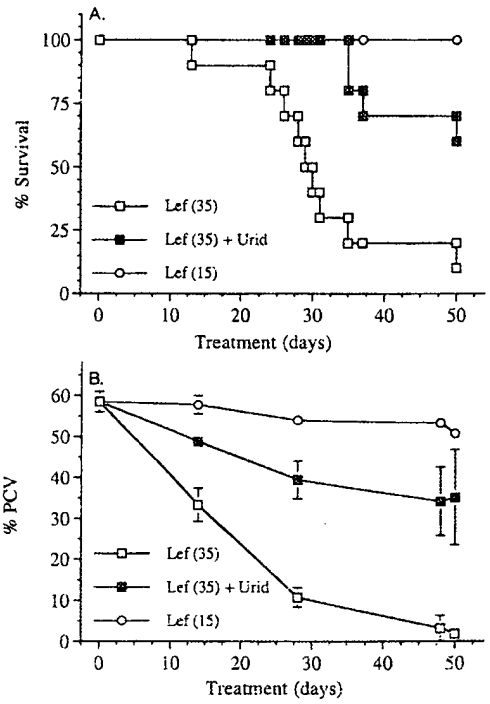

FIGURE 6. (A) Survival of Lewis recipients after treatment with leflunomide (Lef; 15 or 35 mg/kg/day) alone or in combination with uridine (Urid; 500 mg/kg/day). (B) The hematocrits in Lewis recipients after treatment with leflunomide (Lef; 15 or 35 mg/kg/day) alone or in combination with uridine (Urid; 500 mg/kg/day). Data are presented as mean PCV of 10 rats±SE.

vented changes in the small bowel in the three surviving Lewis rats treated with leflunomide and uridine for ≥75 days (Fig. 7C).

Toxicity in the liver was characterized by fatty degeneration, atrophy, and necrosis of the hepatocytes in the central lobular regions (Fig. 7B). These changes were completely abrogated with the co-administration of uridine, and the findings of the histological analysis of the liver seemed normal (Fig. 7D).

DISCUSSION

When relating the *in vitro* activity of a drug to its *in vivo* activity, it is usually necessary to assess the drug levels in the blood and tissue, at doses known to modify the function of the target organ system. With leflunomide, two *in vitro* activities are known to exist at two distinct concentrations: inhibition of *de novo* pyrimidine synthesis and selective inhibition of tyrosine kinases. Either of these activities might account for the immunomodulatory activity of leflunomide. To individually assess these activities *in vivo* requires that at least one of the activities be controlled. Uridine can be used as an antidote for inhibition of *de novo* pyrimidine synthesis; thus, we first conducted a pharmacokinetic analysis of serum A77 1726 after the administration of leflunomide, and of serum uridine and tissue nucleotide triphosphate levels after the administration of uridine.

After a single oral administration of leflunomide (5, 15, and 35 mg/kg), we observed a linear dose-dependent relationship between the dose administered and the concentration of A77 1726 in the serum. Consistent with previous studies, the increase in A77 1726 levels in the serum was relatively slow (Ka=1.97 to 0.484 mL · min/kg) and peak levels were reached in 6–8 hr, irrespective of the dose (*28*, *29*). After the administration of a single, i.p. dose 500 mg/kg of uridine in Lewis rats, increased levels of serum uridine were detected almost immediately and the peak concentration of uridine in the serum, 384.1±53.5 μM/L, was observed within 15 min (*28*, *29*). However, uridine plasma levels returned to normal by 4 hr, indicating that the pharmacokinetics of uridine are significantly different from that of A77 1726. The levels of UMP and CTP in the spleen, lymph nodes, and liver of Lewis rats were elevated within 1 hr after the administration of 500 mg/kg uridine. In contrast to rapid elimination of serum uridine, the levels of UTP and CTP in the spleen, liver, and lymph nodes remained elevated for 6–12 hr. Our data further suggest a hierarchy in the duration of elevated pyrimidine nucleotides in the spleen>liver>lymph nodes, perhaps reflecting the ability of different tissues or cells to salvage uridine and the availability of serum uridine (*30*, *31*). A

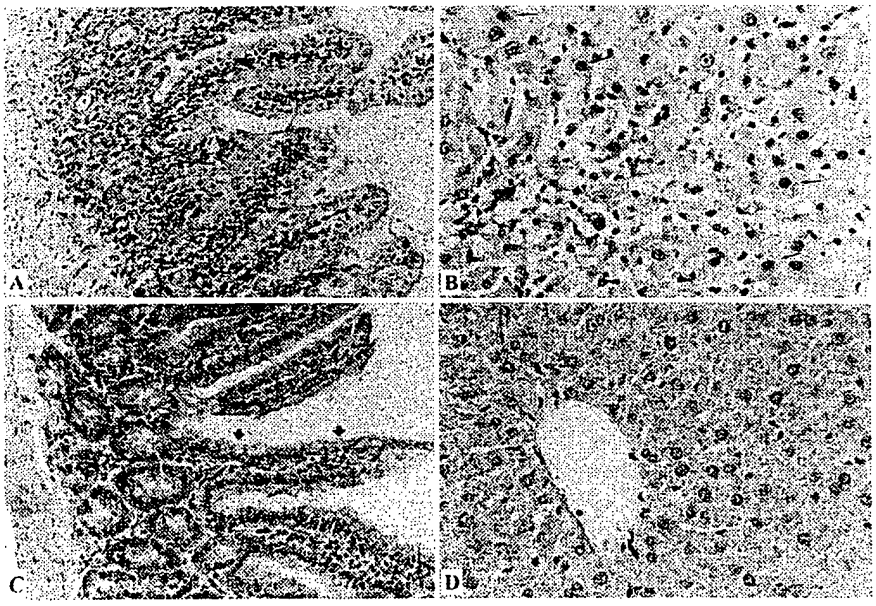

FIGURE 7. Histological examination of the effects of leflunomide (35 mg/kg/day) monotherapy (A, B), or in combination with uridine (C, D) in the small bowel (A, C), and liver (B, D). Liver and small bowel were stained with hematoxylin and eosin.

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critical role of the spleen in the development of cellular and antibody responses is suggested by the central position of the spleen in blood circulation and the large numbers of lymphocytes migrating. Thus, it is noteworthy that the pharmacokinetic findings of UTP levels after uridine administration in the spleen is most similar to that of serum A77 1726 after administration of leflunomide.

We next examined the effects of uridine on the immunosuppressive activity and toxicity of leflunomide in Lewis rats receiving with brown Norway or Golden Syrian hearts. On the basis of three criteria: survival, histological examination of the allograft, and titers of alloreactive antibodies, we conclude that the ability of leflunomide to control allograft rejection seems to be only minimally affected by uridine co-administration. It is interesting that the lymphoid areas in the spleen that were significantly reduced in leflunomide-treated rats receiving allograft hearts were significantly reversed by uridine co-administration. We, therefore, speculate that the immunosuppressive effects of leflunomide in this allotransplantation model are independent of the DHO-DHase-dependent antiproliferative effects of leflunomide. In a second allograft model (Balb/c into C3H), uridine also had modest effects on the immunosuppressive activity of leflunomide. These data are consistent with the conclusion that the mechanism by which leflunomide controls alloreactivity is largely independent of inhibition of pyrimidine synthesis *in vivo*.

The modest effect of uridine in this allograft model contrasts with our *in vitro* data that indicate that uridine can completely antagonize the antiproliferative activity of the active metabolite of leflunomide, A77 1726, when it is used at concentrations that are ≤ 25 μM (*9*). However, we had noted that uridine only partially reversed the antiproliferative effects of A77 1726 when the concentrations were ≥ 50 μM, and had no effect of the ability of A77 1726 to inhibit T cell cytotoxic activity (*9*). Because A77 1726, at IC_{50} of ≥ 50 μM, inhibits tyrosine phosphorylation in lymphocytes, we had hypothesized that the immunosuppressive activity at ≥ 50 μM A77 1726, and in the presence of uridine, results from inhibition of tyrosine phosphorylation (*6*, *9*, *32*).

Single-dose pharmacokinetic studies of rats treated with leflunomide at 5, 15, and 35 mg/kg/day indicated that peak concentrations of A77 1726 in the sera were 77.5, 266.1 and 478.8 μM, respectively, whereas the 24 trough concentrations are 4.9, 6.3, and 125 μM, respectively. Additionally, single-dose pharmacokinetic studies of mice treated with leflunomide at 35 mg/kg/day indicates that the peak concentration of A77 1726 in the sera is 518.2 μM, whereas the 24 hr trough concentration is 227.5 μM (data not shown). Therefore, the inability of uridine to counter the effects of leflunomide in this allograft model is consistent with the *in vitro* observations that uridine cannot reverse the immunosuppressive activities of higher doses of A77 1726 *in vitro* (*9*). We, thus, conclude that the primary mode of immunosuppression by leflunomide in this allograft model may be related to the inhibition of tyrosine phosphorylation and that the inhibition of *de novo* pyrimidine synthesis is of secondary importance.

In contrast to the modest effect of uridine in the allograft model, the ability of uridine to antagonize the immunosuppressive activity of 15 mg/kg/day of leflunomide in the xenograft model is very convincing. In the leflunomide monotherapy groups (15 mg/kg/day), the xenografts survive for a mean of >63 days. There was minimal IgM deposition in the xenograft on day 10 but significant IgM at the time of rejection or on day 75 after the transplant. In the combination therapy group, the xenografts were rejected in 8–10 days, and rejection was associated with extensive deposition of IgM in the xenograft. At the higher dose of leflunomide (35 mg/kg/day), the effect of uridine was more modest and the xenograft hearts were still beating at the time the rats were killed (day 34 or day 75 after the transplant). A histological examination of the xenografts from the combination treatment group (leflunomide [35 mg/kg/day] plus uridine) revealed increased signs of inflammation, chronic rejection, and IgM deposition, compared with the 35 mg/kg/day leflunomide monotherapy group. These observations suggest that inhibition of *de novo* pyrimidine synthesis is an important part of immunosuppressive therapy in the xenotransplantation model. It further suggests that insufficient uridine is not the explanation for our observations in the allotransplantation model.

The contrasting effect of uridine on the immunosuppressive activity of leflunomide may result from different mechanisms of rejection in the allograft versus the xenograft model. Acute xenograft rejection is dependent on the rapid production of xenoreactive IgM; in contrast, acute allograft rejection is a T-cell dependent process (*17*, *33*). Therefore, it is possible that the control of B cell function in xenograft rejection by leflunomide depends more on the inhibition of pyrimidine synthesis, whereas the control of T cells by leflunomide may be more dependent on the inhibition of tyrosine phosphorylation. *In vitro* observations suggest that B cells may be more susceptible to the effects of inhibition of *de novo* pyrimidine synthesis than T cells (*9*, *10*, *34–36*).

A second goal of these studies was to investigate whether uridine could be used to control leflunomide-induced toxicity. The most consistent symptoms in Lewis rats treated with 35 mg/kg/day of leflunomide were severe anemia and diarrhea resulting in weight loss, dehydration and, ultimately, death. Uridine was able to significantly reduce the anemia and prolong the survival of the Lewis rats. Autopsies revealed liver necrosis and pathological changes in the small bowel in rats treated with 35 mg/kg/day leflunomide. Most of these changes were significantly reversed by uridine co-administration, suggesting that the toxicity of the liver and small bowel, and the inhibition of hematopoiesis, is largely caused by inhibition of pyrimidine synthesis.

In summary, we report that uridine had minor effects on the immunosuppressive activity of leflunomide in the allograft model, and a more significant effect in the xenograft model. Thus, it seems that the mechanism of immunosuppression by leflunomide *in vivo* is complex and may be affected by at least the following four factors: the type and vigor of the immune response; the availability of uridine for salvage by proliferating lymphocytes; the species-specific efficacy by which leflunomide inhibits the activity of dihydroorotate dehydrogenase, and the levels of A77 1726 *in vivo*.

Acknowledgments. This work was funded in part by a grant from the National Institutes of Health (AI34061).

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Uridine Reverses the Toxicity of 3'-Azido-3'-Deoxythymidine in Normal Human Granulocyte-Macrophage Progenitor Cells In Vitro without Impairment of Antiretroviral Activity

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We evaluated the effects of natural purine and pyrimidine nucleosides on protection from or reversal of 3'-azido-3'-deoxythymidine (AZT) cytotoxicity in human bone marrow progenitor cells by using clonogenic assays. The selectivity of the "protection" or "rescue" agents was examined in evaluating the antiretroviral activity of AZT in combination with these modulating agents and of AZT alone. Following exposure of human granulocyte-macrophage progenitor cells for 2 h to 5 μM AZT (70% inhibitory concentration), increasing concentrations of potential rescue agents were added. Cells were cultured, and colony formation was assessed after 14 days. At concentrations of up to 50 μM no natural 2'-deoxynucleosides, including thymidine, were able to reverse the toxic effects of AZT. Dose-dependent reversal was observed with uridine and cytidine, and essentially complete reversal was achieved with 50 μM uridine. In the protection studies, 100 μM thymidine almost completely antagonized the inhibition of granulocyte-macrophage colony formation produced by 1 μM AZT (50% inhibitory concentration), and 50 μM uridine effected 60% protection against a toxic concentration of AZT (5 μM) (70% inhibitory concentration). The antiretroviral activity of AZT in human peripheral blood mononuclear cells, assessed by reverse transcriptase assays, was substantially decreased in the presence of thymidine, whereas no impairment of suppression of viral replication was observed in the presence of uridine in combination with AZT at a molar ratio (uridine/AZT) as high as 10,000. This demonstration of the capacity of uridine to selectively rescue human bone marrow progenitor cells from the cytotoxicity of AZT suggests that use of uridine rescue regimen with AZT may have potential therapeutic benefit in the treatment of acquired immunodeficiency syndrome.

3'-Azido-3'-deoxythymidine (AZT), a pyrimidine nucleoside synthesized two decades ago by Horwitz et al. (7), has recently been shown to transiently improve certain immunological functions in some patients with acquired immunodeficiency syndrome (AIDS) (3), resulting in a decrease in the incidence of opportunistic infections and prolonging survival. The antiretroviral effects of AZT are probably based upon its conversion through cellular kinases to AZT triphosphate, which binds to reverse transcriptase and thereby inhibits viral DNA synthesis by chain termination (4). Although AZT selectively inhibits the replication of human immunodeficiency virus type 1 (HIV) (10), its applications in preliminary clinical trials (11, 15) were limited by expressions of bone marrow toxicity. Consistent with these expressions we recently reported (12) that continuous exposure to AZT for 14 days effected a dose-dependent inhibition of human granulocyte-macrophage CFU (CFU-GM) and erythroid burst-forming unit colonies, the 50% inhibitory concentrations being 0.9 ± 0.1 and 2.4 ± 0.4 μM for the respective colonies. Several pharmacologic approaches are potentially available to improve the chemotherapeutic selectivity of AZT. Such an improvement can be theoretically obtained with synergistic combination chemotherapy, as recently demonstrated with recombinant human granulocyte-macrophage colony-stimulating factor (5) and alpha A interferon (6), potentially allowing a reduction in AZT concentrations. Selective "protection" or "rescue" combinations may also achieve this goal. In this approach, the modulating agents are used at a time and a dosage that counteract (protection) or reverse (rescue) the toxic effects in the host cell without interfering with the chemotherapeutic activity. This concept has been previously used in cancer chemotherapy with methotrexate and its "antidote," leucovorin (2), and more recently for treating protozoan infections with trimetrexate and leucovorin in AIDS patients (1).

The present report relates the results of an evaluation of the capacities of various natural nucleosides to protect or to reverse AZT toxicity in human host cells. The selectivity of the metabolic modulations was assessed by evaluating their antiretroviral activity in comparison with that of AZT alone in HIV-infected cells.

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MATERIALS AND METHODS

Chemicals. Purine and pyrimidine ribonucleosides and deoxyribonucleosides were purchased from Sigma Chemical Co., St. Louis, Mo. AZT was synthetized in our laboratory by the procedure of Lin and Prusoff (8) and had a purity of >99%, as assessed by high-pressure liquid chromatography. The structure of the compound was confirmed by proton nuclear magnetic resonance, ^{13}C nuclear magnetic resonance, and infrared spectroscopy. Other chemicals were of the highest quality commercially possible.

Virus strains. HIV strain LAV was obtained from the

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Centers for Disease Control, Atlanta, Ga., and propagated as recently described (13).

Preparation of cells. Human bone marrow cells were collected by aspiration from the posterior iliac crest of normal healthy volunteers and treated with heparin, and the mononuclear population was separated by Ficoll-Hypaque gradient centrifugation. Cells were washed twice in Hanks balanced salt solution and counted with a hemacytometer, and their viability was >98%, as assessed by trypan blue exclusion. Peripheral blood mononuclear (PBM) cells were obtained from the whole blood of healthy HIV- and hepatitis B virus- seronegative volunteers and collected by single-step Ficoll-Hypaque discontinuous gradient centrifugation.

Assay of CFU-GM for drug cytotoxicity and rescue or protection studies. The culture assay of CFU-GM was performed by a bilayer soft-agar method as recently described (12). McCoy 5A nutrient medium supplemented with 15% dialyzed fetal bovine serum (heat inactivated at 56°C for 30 min) (GIBCO Laboratories, Grand Island, N.Y.) was used in all experiments. This medium completely lacked thymidine and uridine.

In the rescue studies, mononuclear cells (10^5/ml) were exposed for 2 h at 37°C in 5 ml of McCoy 5A nutrient medium to 5 μM AZT, corresponding to a 70% inhibitory concentration. At the end of the 2-h incubation period, cells were washed twice with fresh cold incubation medium to wash out the AZT. Cells were subsequently cloned in 0.3% agar in the presence of increasing concentrations of the modulating compound or in medium alone (control). After 14 days of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air, colonies (≥50 cells) were counted by using an inverted microscope.

In the protection studies, AZT (1 or 5 μM) and either medium (control) or various concentrations of thymidine or uridine were added simultaneously. Cells were exposed continuously under these conditions for 14 days, and colonies (≥50 cells) were then scored. The toxicity of each purine and pyrimidine analog investigated in these studies (see Table 1) was assessed by continuous exposure for 14 days by the same technique.

Anti-HIV assay based on reverse transcriptase activity. After phytohemagglutinin stimulation for 3 days, PBM cells (5×10^5/ml) were infected with HIV strain LAV at a concentration of about 100 50% tissue culture infective doses per ml and cultured in the presence of various concentrations of AZT alone or in combination with either uridine or thymidine. The virus was allowed to adsorb for 45 min, and then drugs (AZT and uridine or thymidine) were added. A virus control (no drug) and a cell control (no virus or drug) were also included. On day 5 after infection, clarified supernatant fluids were centrifuged in a Beckman 70.1 Ti rotor at 40,000 rpm for 30 min. The concentrated, disrupted virus was subjected to reverse transcriptase assays as recently described by Spira et al. (13). Antiretroviral efficacy was determined by calculating the percent reduction in reverse transcriptase activity observed in drug-treated, virus-infected cultures as compared with enzyme activity in virus-infected control cultures.

RESULTS

Effect of a short exposure (2 h) of normal human bone marrow progenitor cells to AZT on colony growth. Initial experiments were designed to establish the concentration dependence of human bone marrow progenitor cell toxicity produced by AZT after 2 h of drug exposure. Normal human bone marrow cells were incubated at 37°C for 2 h with various concentrations of AZT, and cells were washed twice prior to plating. Cell viability was determined by soft-agar cloning and measurement of colony formation after drug treatment as described in Materials and Methods. After 2 h of drug exposure, the toxic effects of AZT (Fig. 1) were quite similar to those recently reported for these cells after continuous exposure (14 days) to AZT (12), suggesting that the toxicity of AZT in human bone marrow progenitor cells in vitro is probably mediated through early effects.

Ability of purine or pyrimidine derivatives to reverse the toxicity of AZT in human bone marrow cells. The effects of

TABLE 1. Reversal of AZT cytotoxicity in human bone marrow progenitor cells by naturally occurring purine and pyrimidine nucleosides

Compound and concn (μM)	Survival (% of untreated control)[a] in the presence of compound:	
	Alone	With AZT (5 μM)[b]
Thymidine		
0	100	22.8 ± 7.8
5	ND[c]	24.6 ± 7.1
10	85.4 ± 3.1	24.6 ± 8.4
50	84.7 ± 14.1	17.7 ± 7.5
Cytidine		
0	100	22.8 ± 7.8
5	ND	25.3 ± 12.4
10	94.0 ± 7.3	23.5 ± 5.3
50	93.3 ± 6.2	50.0 ± 14.0[d]
Uridine		
0	100	22.8 ± 7.8
5	ND	24.5 ± 13.6
10	85.4 ± 3.1	43.2 ± 14.4[e]
50	84.7 ± 14.1	100.7 ± 20.3[d]
100	ND	85.6 ± 10.4[d]
2'-Deoxyuridine		
0	100	33.0 ± 16.0
5	ND	37.0 ± 10.4
10	100 ± 8.0	34.0 ± 6.0
50	91.3 ± 2.3	35.0 ± 10.5
2'-Deoxyadenosine		
0	100	33.0 ± 16.0
5	ND	36.0 ± 7.0
10	66.7 ± 5.5	37.3 ± 15.5
50	63.3 ± 6.4	42.3 ± 8.1
2'-Deoxyguanosine		
0	100	33.0 ± 16.0
5	ND	33.6 ± 16.2
10	83.4 ± 3.8	30.0 ± 6.2
50	ND	40.0 ± 6.0
2'-Deoxycytidine		
0	100	33.0 ± 16.0
5	ND	28.0 ± 7.0
10	82.7 ± 12.6	34.0 ± 10.4
50	94.6 ± 4.6	35.0 ± 4.6

[a] Each value represents the mean ± standard deviation in at least three experiments with at least three different marrow donors.
[b] Cells were incubated with AZT (5 μM) for 2 h, washed twice, and cultured for clonal growth for 2 weeks in the presence of purine or pyrimidine analogs.
[c] ND, Not determined.
[d] $P < 0.001$ as compared with the control.
[e] $P < 0.01$ as compared with the control.

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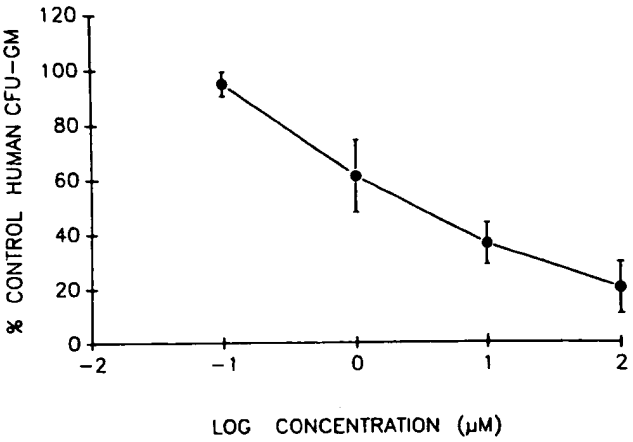

FIG. 1. Effects of a short exposure (2 h) of normal human bone marrow progenitor cells to increasing concentrations of AZT. Each point represents the mean ± standard deviation of at least six experiments with different marrow donors.

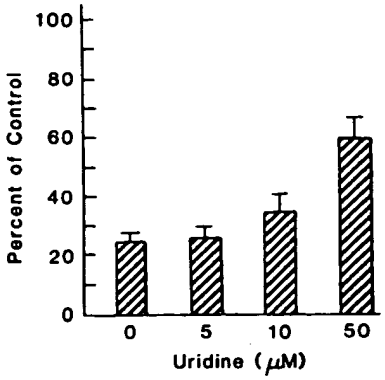

FIG. 2. Effect of AZT (5 μM) on hematopoietic colony growth by normal human bone marrow progenitor cells in the presence of various concentrations of uridine. Columns represent the mean percentage of inhibition of CFU-GM colonies in three separate experiments; bars represent the standard deviation. The mean number of colonies in the control plates (without AZT and uridine) was 60 ± 5 CFU-GM per 10^5 cells. P was <0.01 for 10 μM uridine and <0.001 for 50 μM uridine as compared with the control.

adding purine or pyrimidine nucleoside analogs to human hematopoietic progenitor cells following 2 h of exposure to 5 μM AZT (70% inhibitory dose) are shown in Table 1. No natural purine or pyrimidine 2'-deoxynucleosides (2'-deoxyuridine, 2'-deoxyadenosine, 2'-deoxyguanosine, 2'-deoxycytidine, and thymidine) up to a concentration of 50 μM reversed the toxic effects of AZT, nor was reversal achieved with 2'-deoxynucleoside concentrations of up to 200 μM (data not shown). In contrast, nontoxic concentrations of uridine or cytidine effected a significant and dose-dependent reversal of AZT toxicity. Essentially complete reversal was achieved with 50 μM uridine, and no significant difference was detected in the presence of higher concentrations of uridine (100 μM). At concentrations of 50 μM the rescue effect of cytidine was less than that of uridine. The reversal of AZT toxicity by cytidine probably depends on the conversion of cytidine to uridine by cytidine deaminase, a requirement that could explain the difference in the rescue potencies of the two pyrimidine derivatives.

Protection from AZT toxicity by uridine in human bone marrow cells. Simultaneous exposure to 5 μM AZT (70% inhibitory dose) and various concentrations of uridine was also investigated in our studies to assess whether uridine could protect human bone marrow progenitor cells from AZT toxicity when both drugs were present throughout the experiment. Figure 2 illustrates the effects of 5 μM AZT on hematopoietic colony growth of normal human bone marrow progenitor cells in the presence of 5 to 50 μM uridine. Dose-dependent protection was observed, with 50 μM uridine effecting approximately 60% protection in the presence of a toxic concentration of AZT (5 μM).

Protection from AZT toxicity by thymidine in human bone marrow cells. Since thymidine counteracts the antiretroviral activity of AZT in ATH8 cells (10) and both AZT and thymidine appear to share the same activating enzymes (i.e., thymidine and thymidylate kinases) (4), protection from AZT toxicity by thymidine was investigated in human bone marrow progenitor cells. Human granulocyte-macrophage precursor cells, grown in soft agar, were exposed continuously for 14 days to 1 μM AZT (50% inhibitory dose) and 10 to 100 μM thymidine. The latter agent antagonized the inhibition of colony formation by AZT in a dose-dependent manner, almost complete protection being effected by 100 μM thymidine (Fig. 3).

Effect of thymidine and uridine on the antiretroviral activity of AZT in HIV-infected human PBM cells. The effects of thymidine and uridine on the capacity of AZT to inhibit HIV replication in human PBM cells was evaluated (Table 2). Essentially full protection against HIV production was achieved with AZT alone at a concentration of approximately 0.01 μM, in agreement with recently published data (9). The presence of thymidine led to a substantial loss of the antiretroviral activity of AZT, as reported previously (10). A concentration of 10 μM thymidine reduced the inhibition of HIV by 0.01 μM AZT by approximately 50%, and the anti-HIV activity of 0.01 μM AZT was essentially abolished by 100 μM thymidine. In contrast, concentrations of uridine of up to 100 μM and in combination with AZT at a molar

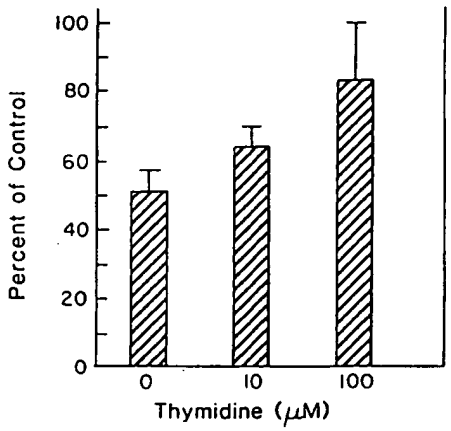

FIG. 3. Effect of AZT (1 μM) on hematopoietic colony growth by normal human bone marrow progenitor cells in the presence of various concentrations of thymidine. Columns represent the mean percentage of inhibition of CFU-GM colonies in three separate experiments; bars represent the standard deviation. The mean number of colonies in the control plates (without AZT and thymidine) was 80 ± 20 CFU-GM per 10^5 cells.

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TABLE 2. Effect of thymidine and uridine on AZT antiretroviral activity in HIV-infected human PBM cells

Treatment and concn (μM)	% Inhibition on day 5[a] (corrected)
AZT	
0.001	0
0.01	81.9
0.1	96.6
Thymidine	
10	0
100	0
Uridine	
10	7.3
100	5.1
AZT-thymidine	
0.01-0.1	81.4
0.01-1	85.0
0.01-10	52.6
0.01-100	4.1
AZT-uridine	
0.01-10	75.1
0.01-100	63.2
0.1-10	96.7
0.1-100	96.1

[a] The mean of triplicate counts (± standard deviation) for the virus control was 234,780 ± 26,600 dpm/ml (equivalent to an approximate incorporation of 3.6 pmol of dTMP into the acid-unsoluble product). Counts for the blank and negative control (no virus or drug) were 355 and 1,535 dpm, respectively. A positive control for the reverse transcriptase assay was also included (count, 313,000 dpm).

ratio (uridine/AZT) as high as 10,000 had no effect on the antiretroviral activity of AZT. Cell viability and growth, as assessed by trypan blue exclusion and with a Coulter Counter, were similar among the cultures, and no toxicity was observed in the presence of 100 μM uridine (Table 3).

TABLE 3. Effect of AZT and uridine alone or in combination on human PBM cell growth and cell viability[a]

Treatment and concn (μM)	Growth (% of untreated control)	Viability (% of untreated control)
AZT		
0.1	102.9	113.2
1	100	90.6
10	81.2	100
100	79.4	103.1
Uridine		
1	100	109.4
10	94.1	100
100	87.0	100
AZT-uridine		
1-1	97.1	78.8
1-10	105.9	72.5
1-100	78.1	78.2

[a] The mean numbers of human PBM cells (± standard deviation) in the cell growth and viability studies were (3.2 ± 0.6) × 10^6 cells per ml and 3.4 ± 0.3 cells per ml, respectively. Cells were stimulated with phytohemagglutinin for 2 days and subsequently exposed for 5 days to various concentrations of AZT or uridine alone or in combination.

These data suggest that, unlike thymidine, uridine does not interfere with the uptake and/or metabolic activation of AZT in HIV-infected human PBM cells or any other mechanism(s) by which AZT inhibits HIV replication.

DISCUSSION

In a previous report from this laboratory, we demonstrated that AZT directly suppressed human hematopoietic colony growth in a dose-dependent manner by direct interaction with CFU-GM and erythroid burst-forming unit progenitor cells (12). These findings were consistent with the observation that anemia and neutropenia were the major adverse effects of AZT administration to AIDS patients (11, 15). In an effort to obviate this untoward effect of AZT, we have sought to determine in the present study whether natural nucleosides can protect against or reverse the toxicity of AZT in human bone marrow progenitor cells without affecting the antiretroviral activity of AZT. As reported here, thymidine can counteract or protect against the toxicity of AZT in normal human bone marrow cells, but it also antagonizes the antiretroviral activity of AZT (Table 2). These data probably reflect a decrease in the formation of AZT triphosphate, since both AZT and thymidine utilize the same activating enzymes (i.e., thymidine and thymidylate kinases) to exert their pharmacologic action. It is particularly important that thymidine, even at concentrations of up to 100 μM, was not able to reverse the toxic effects of 5 μM AZT (70% inhibitory concentration) for human granulocyte-macrophage precursor cells. This result suggests that the administration of thymidine sequentially with AZT in vivo probably will not prevent its toxic effects, as previously speculated (11).

Our experiments also showed that the hematopoietic toxicity of AZT was consistently reversed by uridine and to a lesser extent by cytidine. The inhibition of CFU-GM colony formation at an AZT concentration of 5 μM was essentially reversed when 50 μM uridine was added to the cultures. The percentage of rescue of CFU-GM colony formation was proportional to the concentration of uridine, suggesting that the reversal was a competitive process. The same concentration of cytidine only partially reversed the toxic effects of AZT on colony formation. In these studies, cytidine probably acted through its conversion to uridine by cytidine deaminase, explaining the quantitative difference in the rescue between the two pyrimidine derivatives. Uridine was shown also to protect normal human bone marrow progenitor cells from AZT toxicity, and 60% protection was achieved when cells were exposed to both 5 μM AZT and 50 μM uridine for 14 days.

No difference in the inhibition of viral replication was observed in HIV-infected PBM cells when uridine was combined with AZT at different molar ratios as compared with AZT alone. Therefore, the combination of AZT and uridine appears to selectively reverse the hematopoietic effects of AZT without decreasing its antiretroviral activity. Uridine pharmacokinetic and toxicity studies have been recently reported in humans (14), and "rescuing" concentrations of uridine may be achieved in vivo, with a tolerable toxicity, making this combination potentially suitable for the treatment of AIDS. A potential mechanism(s) which may account for the reversal of or protection from AZT cytotoxicity by uridine in human bone marrow cells is unclear, and further investigations are currently in progress.

In summary, the high degree of selectivity of the uridine rescue between human bone marrow progenitor cells and

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HIV-infected cells suggests that the combined use of AZT and uridine may be of importance in the treatment of AIDS. Although the results of the present in vitro studies must be cautiously extended to the clinical situation, the possible use of uridine for rescue may have a potential therapeutic benefit in that the antiretroviral activity of AZT is not affected while the host toxicity of AZT is minimized. This novel strategy for modulating AZT therapy deserves further biochemical and/or pharmacologic investigations, which may lead to carefully controlled clinical evaluations.

ACKNOWLEDGMENTS

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Uridine in the prevention and treatment of NRTI-related mitochondrial toxicity

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Long-term side effects of antiretroviral therapy are attributed to the mitochondrial (mt) toxicity of nucleoside analogue reverse transcriptase inhibitors (NRTIs) and their ability to deplete mtDNA. Studies in hepatocytes suggest that uridine is able to prevent and treat mtDNA depletion by pyrimidine NRTIs [zalcitabine (ddC) and stavudine (d4T)] and to fully abrogate hepatocyte death, elevated lactate production and intracellular steatosis. Uridine was also found to improve the liver and haematopoietic toxicities of zidovudine (AZT), which are unrelated to mtDNA depletion, and to prevent neuronal cell death induced by ddC. Most recently, uridine was found to prevent the onset of a lipoatrophic phenotype (reduced intracellular lipids, increased apoptosis, mtDNA depletion and mt depolarization) in adipocytes incubated long-term with d4T and AZT. Various steps of mt nucleoside utilization may be involved in the protective effect, but competition of uridine metabolites with NRTIs at polymerase γ or other enzymes is a plausible explanation. Pharmacokinetic studies suggest that uridine serum levels can be safely increased in humans to achieve concentrations which are protective *in vitro* (50–200 μM). Uridine was not found to interfere with the antiretroviral activity of NRTIs. Mitocnol, a sugar cane extract which effectively increases uridine in human serum, was beneficial in individual HIV patients with mt toxicity and is now being tested in placebo-controlled randomized trials. Until these data become available, the risk–benefit calculation of using uridine should be individualized. The current safety data justify the closely monitored use of uridine in individuals who suffer from mt toxicity but who cannot be switched to less toxic NRTIs.

Introduction

More than 8 years after the widespread introduction of highly active antiretroviral therapy (HAART), it has become clear that antiretroviral drugs have long-term effects on organs and body metabolism. Nucleoside reverse transcriptase inhibitors (NRTIs) within the antiretroviral cocktail are associated with hyperlactataemia and organ toxicities such as damage to the liver, peripheral nerves and skeletal muscle. The choice of NRTI also determines an individual's risk of developing lipoatrophy, a clinically irreversible loss of subcutaneous tissue. The main mechanism of these NRTI-related side effects has been identified as mitochondrial (mt) toxicity [1–7].

Pathogenesis of NRTI-related mt toxicity

NRTIs are activated by triphosphorylation and then they inhibit polymerase γ, the enzyme which replicates mtDNA [3,8]. Polymerase γ inhibition is a result of several distinct steps [3]. The first step involves competition of NRTI triphosphates with the natural nucleoside triphosphates. If this competition is successful, the NRTIs are incorporated into the nascent mtDNA strand. This second step causes chain termination. As a result of polymerase γ impairment, mtDNA depletion (a quantitative reduction of the mtDNA copy number) ensues. The relative potency of activated nucleoside triphosphates to inhibit polymerase γ is not the same among all NRTIs. *In vitro* data indicate a relatively strong inhibitory effect of the 'd-drugs', that is, zalcitabine (ddC), didanosine (ddI) and stavudine (d4T), whereas abacavir, emtricitabine, lamivudine and tenofovir do not impair mtDNA replication in clinically relevant concentrations [3,8,9].

Zidovudine (AZT) is a special case because this NRTI is a mt toxin despite the fact that AZT triphosphate only has a low potency to affect polymerase γ and mtDNA content in clinically relevant and cytotoxic concentrations, at least in proliferating cells [8–11]. On one hand, the mt toxicity of AZT may, in part, involve binding to adenylate kinase (an enzyme involved in ATP formation) and inhibition of the mt ADP/ATP translocator [12–14]. These mechanisms may explain why some toxicities have been observed relatively early after AZT exposure [9,13]. On the other hand, mtDNA depletion has indeed been

observed with AZT *in vivo* [6,15–17]. Two observations may explain why mtDNA depletion may also occur in the absence of direct polymerase γ inhibition. Firstly, it has been shown *in vivo* that some of the administered AZT can be non-enzymatically converted into d4T, and thus a stronger polymerase γ inhibitor [18]. Secondly, mtDNA depletion may result from a another mechanism, namely from AZT-mediated inhibition of thymidine kinase (TK) type 2 [19]. This TK is expressed in mitochondria and responsible for the intramitochondrial phosphorylation of pyrimidine nucleosides (deoxythymidine, deoxycytidine and deoxyuridine). In non-replicating cells, the cytosolic TK type 1 (TK1) is down-regulated, making the pyrimidine supply for mtDNA synthesis dependent on the activity of TK2. Such reduced supply of the normal deoxypyrimidine phosphates limits mtDNA replication, especially in skeletal muscle, as evidenced by a mt myopathy in subjects carrying TK2 mutations [20].

As mtDNA encodes for subunits of the mt respiratory chain, mtDNA depletion therefore results in respiratory chain dysfunction.

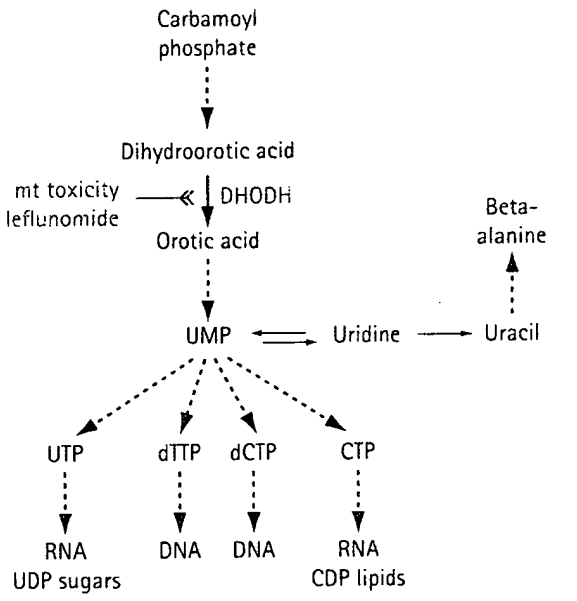
Any respiratory chain dysfunction may promote electron leakage in the mt matrix and thus the generation of reactive oxygen species (ROS). Such increased ROS formation may then in turn damage the lipid architecture of the mt membrane, attack respiratory chain proteins or damage polymerase γ and mtDNA itself, thereby closing several vicious circles that promote even more ROS formation [21,22]. There is also evidence for additional mechanisms of ROS formation [1]. Markers of oxidative damage and hereroplasmic mtDNA point mutations have indeed been shown to increase in patients treated with NRTIs [23,24].

Respiratory chain dysfunction also leads to the secondary impairment of several metabolic pathways. Firstly, ATP can no longer be synthesized efficiently through oxidative phosphorylation and glycolysis has to be relied upon. Secondly, the block of NADH utilization in the respiratory chain increases the intracellular NADH/NAD+ ratio. This alteration of the redox status promotes the conversion of pyruvate to lactate and inhibits key enzymes of beta oxidation, resulting in the intracellular accumulation of triglycerides [25].

The mt respiration also has a third important task: an efficient electron-flux through the respiratory chain is essential for the activity of dihydroorotate-dehydrogenase (DHODH; E.C. 1.3.99.11), an enzyme located in the inner mt membrane and necessary for the *de novo* synthesis of all (intramitochondrial and intracytoplasmic) pyrimidines [26]. This is because DHODH catalyses the oxidation of dihydroorotate to orotate from which uridine monophosphate (UMP) and intracellular pyrimidines are synthesized (Figure 1). A defect in the respiratory chain therefore results in pyrimidine depletion. The indirect inhibition of DHODH by NRTI-related mt toxicity is likely to be similar to those caused by direct DHODH inhibitors [27]. Research into leflunomide [27,28], a direct DHODH inhibitor and a licensed immunosuppressive drug has taught us about the *in vitro* and *in vivo* consequences of DHODH inhibition (Figure 2). The depletion of UMP and derived intracellular pyrimidines activates p53 and its immediate transcriptional target p21 [27,29]. p53 also regulates the activation of Rb protein and thus of cyclins via phosphorylation [30]. Through this mechanism, the pyrimidine depletion inhibits the transition to the S-phase of the cell cycle and leads to a mitotic arrest in the G1 phase. p53 can also activate the transcription of *Bax* [31] and promote apoptosis. These molecular mechanisms may explain why cells with mtDNA depletion stop dividing and then die.

The importance of the intracellular pyrimidine pools for the survival of cells without a functional respiratory chain is supported by the fact that cells without a single molecule of mtDNA (rho0-cells) are rescued from cell

Figure 1. Simplified scheme of pyrimidine metabolism



The biosynthetic pathway starts with the formation of carbamoyl phosphate. DHODH (an enzyme which is inhibited by respiratory chain dysfunction in mt toxicity of NRTIs and by leflunomide) then catalyses the synthesis of orotate. Orotate is then anabolized to UMP, which can be used to produce RNA, DNA, glycosylation products or membrane constituents. Uridine can be salvaged into UMP by uridine kinase or degraded into beta-alanine, which enters the tricarboxylic acid cycle. Dashed arrows signify pathways involving intermediate metabolites. mt, mitochondrial; CDP, cytidine diphosphate; DHODH, dihydroorotate dehydrogenase; dCTP, dideoxycytidine triphosphate; dTTP, dideoxythymidine triphosphate; UDP, uridine diphosphate; UMP, uridine monophosphate; UTP, uridine triphosphate.

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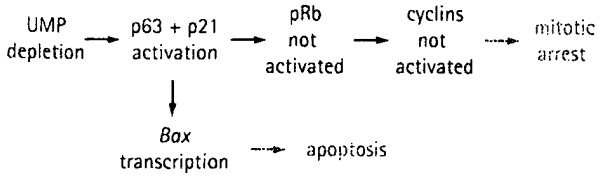
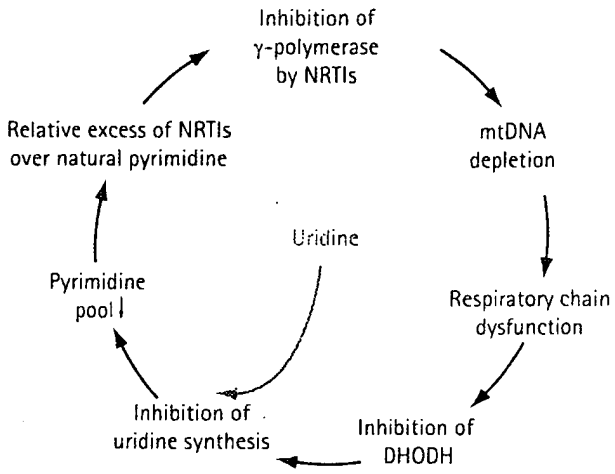
Figure 2. Molecular and functional consequences of diminished intracellular pyrimidine supply



UMP, uridine monophosphate.

Figure 3. Vicious cycle which is hypothesized to contribute to the mt toxicity of antiretroviral pyrimidine d-drugs and which may be abrogated by uridine supplementation



mt, mitochondrial; DHODH, dihydroorotate dehydrogenase; NRTI, nucleoside reverse transcriptase inhibitor.

death and grow virtually normally if the intracellular pyrimidine pools are replenished by substances that can be salvaged into pyrimidines by being converted into UMP distal to DHODH. One such substance that can bypass the block in the *de novo* synthesis of pyrimidines is uridine [32].

Uridine abrogates mt toxicity *in vitro*

The relationship between respiratory chain dysfunction and pyrimidine metabolism makes uridine an attractive candidate to alleviate symptoms of NRTI-related mt toxicity. Early work has demonstrated that neuronal cells exposed to ddC are rescued from death and improve in proliferation and neurite outgrowth if the medium was supplemented with uridine (50 μM) [33]. Uridine in concentrations of 50 μM also completely reversed the haematopoietic toxicity of AZT (5 μM) on normal human granulocyte–macrophage progenitor cells [34]. Similar strategies in mouse models of AZT-induced bone marrow suppression reversed anaemia, leucopoenia, increased peripheral reticulocytes and increased bone marrow cellularity [35]. The mechanism for the beneficial action of uridine on AZT is still unclear. As discussed above, AZT has many effects on cell metabolism [8,19]. It is conceivable that uridine or its derived pyrimidines may compete with AZT for one or several of these metabolic steps or, alternatively, for kinases or transporters responsible for the intramitochondrial presence of triphosphorylated AZT [8,34].

Investigations into a model of d-drug-related hepatotoxicity made the surprising discovery that uridine was not only able to prevent cell death (an expected finding), but also to prevent the onset of a severe mtDNA depletion and thereby normalize the synthesis of mtDNA-encoded respiratory chain subunits. This also normalized the rate of lactate production and the intracellular triglyeride content [36]. Importantly, uridine was only able to improve the mtDNA depletion caused by pyrimidine NRTIs, not that caused by purine analogues such as ddI.

The ability of uridine to antagonize the polymerase γ inhibition by pyrimidine d-drugs may be explained by its ability to disrupt the following vicious circle (Figure 3): as discussed above, polymerase γ inhibition involves competition of NRTIs with the natural nucleotides as a first step. mtDNA depletion, respiratory dysfunction, DHODH inhibition and pyrimidine depletion ensue. The decrease in intracellular pyrimidines most probably allows for a more efficient competition of the exogenous nucleoside analogue at polymerase γ. Thus, a vicious circle is closed and drives the cell into further mtDNA depletion. We hypothesize that this circle is disrupted by supplying uridine as an exogenous source of intracellular pyrimidines. The data also suggest that the ability of uridine to abrogate mt toxicities was proportional to the concentration of uridine [33,34,36], underlining the hypothesis of a competitive process. Alternatively, uridine may compete with antiretrovirals at steps of intracellular NRTI transport and phosphorylation.

Most recently, long-term exposure of adipocytes to d4T (10 μM), ddC (0.2 μM) or AZT (1 μM) was shown to induce a lipoatrophic phenotype consisting of apoptosis, loss of lipids, mtDNA depletion, loss of mtDNA-encoded respiratory chain subunits and disruption of the mt membrane potential [37]. The addition of uridine (200 μM) completely abrogated all these effects on adipocytes.

Notably, uridine was not only able to prevent the onset of mt toxicity but also to treat toxicities that were already established [35,36]. Interestingly, in the absence of uridine it took considerably longer for mtDNA depletion to develop (weeks), than it took for uridine to revert such mt toxicity (days) [36]. This relatively quick therapeutic effect of uridine relative to the more prolonged development of mt toxicities may allow for intermittent uridine dosing in order to 'reset the mitochondrial clock'.

Metabolism, pharmacokinetics and safety of uridine in humans

Normal uridine concentrations range from 3–8 μM in human blood plasma, bone marrow and cerebrospinal fluid [38]. Although uridine is part of our everyday food, diet is not an important source of uridine [39,40]. Clinical studies and animal models suggest that uridine is mostly produced in the liver and that erythrocytes serve as carriers for distributing the uridine throughout the body [38]. Exogenous uridine rapidly disappears from plasma ($t_{1/2}$=2 min), reflecting a concentrative and, under physiological conditions, unsaturated entry into tissue cells, as well as catabolism by the liver [41]. Subsequently, the tissue uridine pools turn over with half-lives of 13 to 18 h [41]. The physiological range of uridine in the human plasma was shown not to completely satisfy the pyrimidine requirements of dividing cells, making some *de novo* synthesis necessary for optimal proliferation [42]. Circulating uridine may nevertheless be of physiological importance by allowing dividing cells to utilize their salvage pathway [42].

Uridine has several metabolic fates in the cell (Figure 1). Exogenous uridine is rapidly incorporated into nucleotides in nucleated cells [43]. Uridine can be converted to dTTP and dCTP, which are used to produce DNA. UTP is used for the synthesis of RNA. UTP can also be converted into CTP, which upon conjugation of lipids forms cellular membrane constituents such as CDP ethanolamine. In the form of UDP sugars, uridine may help in the production of glycogen and in protein glycosylation. Uridine is degraded into beta-alanine, which can enter the tricarboxilic acid cycle (See Figure 1 for abbreviations) [38].

Pharmacokinetic and safety data for uridine were collected in several human Phase 1 and 2 trials. Parental administration of uridine as a 1 h infusion (8 g/m^2) resulted in plasma levels in the millimolar range, far above those required to abrogate NRTI-related mt toxicity [44]. Half-life, volume of distribution (634 ml/kg) and total clearance (4.98 ml/kg/min) of uridine appear to be independent of dose, whereas C_{max} and AUC increase with dose in a linear fashion [44]. In subjects given uridine at doses of 2–12 g/m^2 as a single 1 h infusion, about a fourth of the administered dose was excreted in the urine [44]. Uracil, a uridine catabolite, accounted for 3% of the uridine dose [44]. These intravenous doses were tolerated without side effects. However, transient fever, lasting for 15 min, occurred with higher doses [44,45]. Intermittent infusion schedules with 3 g/m^2/h for 3 h, alternating with a 3-h treatment-free interval, resulted in plasma levels of 138–335 μM during the treatment-free period and were tolerable over the 72-h study period [45]. Parenteral uridine necessitates central venous administration due to the onset of phlebitis if given through a peripheral vein [45]. Uridine can also be administered orally and is generally tolerated without any side effects. However, excessive oral dosing (12 g/m^2) is limited by mild and reversible osmotic diarrhoea due to the relatively poor bioavailability of uridine (7%) [46]. Using other pyrimidine precursors, for example, triacetyluridine [34,47,48] or inhibitors of uridine catabolism or excretion may also be envisaged [35,49,50].

Human uridine serum levels can now be effectively increased with mitocnol, a sugar cane extract with a high content (17%) of nucleosides [51]. 24-hour pharmacokinetic data indicate that consuming 36 g of a powder that contains mitocnol increases human uridine serum levels from baseline values (5.6 μM) to mean uridine serum concentrations (C_{max}) of 152.0 μM [51]. Adverse events were not observed. It is recommended that three sachets of mitocnol are taken on three consecutive days per month, taking into account the relatively quick improvement of mt toxicity from *in vitro* studies.

In summary, the current data indicate that uridine concentrations that are protective *in vitro* can be safely achieved with oral and parenteral dosing. Oral uridine supplementation (150 mg/kg/d) is also recommended and has been safely used long-term in patients with hereditary orotic aciduria, an inborn error of pyrimidine *de novo* synthesis, in which uridine reverses megaloblastic anaemia and other symptoms [52].

Interaction of uridine with antiretroviral nucleotides

If uridine or its metabolites are able to compete with NRTIs at the level of polymerase γ, they may also do so at the level of HIV reverse transcriptase (RT). This poses a theoretical risk for the antiretroviral efficacy of nucleoside analogues. The efficiency of RT inhibition is dependent on the ratio between the normal deoxynucleoside triphosphates and the NRTI triphosphates at the enzyme. For example, mycophenolate mofetil, an inhibitor of purine synthesis, depletes intracellular deoxyguanosine triphosphate and decreases plasma HIV-1 RNA in patients treated with the guanosine

analogue abacavir [53,54]. Uridine may thus theoretically have an opposite effect on RT by increasing the normal deoxypyrimidine triphosphates.

Such an effect of uridine on the antiretroviral activity of pyrimidine analogues was first analysed with regard to AZT [34]. Phenotypic HIV resistance assays demonstrated that uridine did not interfere with viral suppression [34]. Importantly, uridine did not impair the antiretroviral activity even in a 10000-fold molar excess, whereas the maximal therapeutic effects of uridine were already achieved with a 10-fold molar surplus. Investigations in mice also came to the same conclusion [35].

The potential interference of uridine with the antiretroviral activity of NRTIs was also extensively examined in phenotypic HIV resistance assays using nucleoside analogues alone and in combinations [55]. Both X-4 tropic and R-5 tropic HIV isolates were tested and three different detection systems including primary human peripheral blood mononuclear cells were used. Uridine was added in concentrations up to 615 μM. Additionally, in these investigations no effect of uridine on NRTI-mediated viral suppression was detected. Enhancement of the normal intracellular pyrimidine stores therefore does not seem to have a crucial effect on HIV replication.

Taken together, the data suggest that the interaction between uridine and NRTIs in the prevention of mt damage does not necessarily imply a reduced antiretroviral efficacy. Explanations for this selectivity include a separate regulation of mt and cytoplasmic dNTP pools, either at the level of mt transport [56] or by the presence of disparate kinases in both compartments [57]. The differential action of uridine on the mt and antiretroviral replication enzymes may also be caused by differences between the polymerases in selecting the natural nucleotide over the activated NRTI.

Uridine in HIV-infected patients

The selective effect of exogenous uridine on NRTI-inhibited mtDNA replication, but not on NRTI antiretroviral action, implies that HIV-infected patients under treatment with pyrimidine NRTIs and suffering from mt toxicity may benefit from strategies aimed at increasing uridine. Mitocnol was used in an HIV patient with progressive hyperlactataemia, mt steatohepatitis and symptomatic elevation of creatine kinase (CK) under long-term antiretroviral treatment with d4T [58]. The patient was started on mitocnol (three sachets/day for four consecutive days). After 2 weeks, at his next visit, liver and muscle enzymes, as well as the myalgias had improved rapidly, despite unchanged medication. Lactate had normalized after 7 weeks and HIV replication remained below the limit of detection. d4T was then switched to tenofovir with no subsequent clinical or laboratory abnormalities.

Mitocnol is now widely used in Germany. Several and in-part randomized and placebo-controlled clinical trials are currently being conducted to formally analyse whether mitocnol is able to prevent and treat mt toxicities such as lipoatrophy, polyneuropathy, hepatic steatosis and myopathy. Virological failure has not been reported (UA Walker, personal communication).

Perspective

The issues discussed above have several further implications. Uridine supplementation may be used to enhance the therapeutic index of pyrimidine NRTIs and thus allow higher dosing to overcome multidrug resistance in salvage therapy. The available data also suggest the possibility of mtDNA depletion in blood [17,59,60]. If the detected mtDNA depletion in blood secondary to pyrimidine NRTIs indeed also reflects reduced mtDNA copy numbers in lymphocytes and if it exceeded a certain threshold, it would have effects similar to those of the direct DHODH inhibitor leflunomide and of inherited defects in pyrimidine synthesis. From the clinical experience with leflunomide as a licensed immunosuppressive antirheumatic drug, it could then be predicted that the mt toxicity in lymphocytes impairs the proliferation of lymphocytes in response to mitotic stimuli, interferes with CD4 recovery and thus is immunosuppressive. Impaired cell-mediated immune responses and reduced CD4 and CD8 lymphocyte subsets were also observed in several patients with an inherited defect in the *de novo* synthesis of pyrimidines; their immunodeficiency improved upon uridine therapy [52,61]. It was also shown that uridine antagonized the inhibition of lymphocytes by leflunomide [62,63]. Most recent *in vitro* and *in vivo* observations in HIV patients also support the view of mt toxicity as being immunosuppressive [11,64,65]. This also offers the potential for uridine to enhance the CD4 cell recovery of patients under antiretroviral treatment.

Strategies aimed at increasing uridine also improved symptoms in patients harbouring a qualitative defect in mtDNA by carrying inherited mtDNA mutations [66]. In patients with such mtDNA mutations, however, uridine would be predicted to ameliorate only one aspect of respiratory chain dysfunction, namely the consequences of DHODH inhibition and, in contrast with antiretroviral-treated HIV patients, not to improve the underlying mtDNA pathology. Therefore, patients with mtDNA mutations are likely to have a continued defect in ATP synthesis and hyperlactataemia under uridine. Further clinical data on this group of patients are eagerly awaited.

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Many aspects of uridine are still poorly understood. For example, an oral dose of 300 mg three times daily for 6 months also improved diabetic neuropathy in a well-conducted trial [67]. Therapeutic uses of uridine were also proposed in cardiovascular disease, hypertension, liver disease and infertility, among others [38].

Until the data from formal clinical studies are available, the risk–benefit calculation of using uridine in HIV-infected patients should be individualized. The current safety data justify the current use of uridine in individuals suffering from mt toxicity who are closely monitored and who cannot be switched to an antiretroviral regimen with a lower potential of mt toxicity.

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(54) Title: METHODS AND COMPOSITIONS FOR REDUCING TOXICITY ASSOCIATED WITH LEFLUNOMIDE TREATMENT

(57) Abstract: The invention relates to methods and compositions useful in alleviating or reducing toxicity associated with leflunomide administration without reducing its bioactivity, e.g., without reducing its immunosuppressive activity, that is, utilizing a bioavailable pyrimidine compound to ameloriate the toxic effects caused by leflunomide compounds.

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METHODS AND COMPOSITIONS FOR REDUCING TOXICITY ASSOCIATED WITH LEFLUNOMIDE TREATMENT

Leflunomide is an isoxazole derivative which has shown therapeutic potential in a diverse array of disease processes and conditions, e.g., as an antiinflammatory agent, an immunosuppressive agent, an anticancer agent and an antiviral agent.

Leflunomide is currently approved in the United States for use in the treatment of rheumatoid arthritis to reduce joint inflammation. It is marketed under the trademark ARAVA®.

U.S. Patent Nos. 5,624,946 and 5,688,824, incorporated herein by reference in their entirety, report that leflunomide has been used experimentally as an immunosuppressive agent in the treatment and prevention of chronic rejection in xenograft and allograft transplant recipients, both alone and in combination with other immunosuppressive agents.

In addition to data suggesting its value in treating, preventing and reversing acute and chronic rejection, U.S. Patent Application U.S. 2003/0114597, incorporated herein by reference in its entirety, reports that leflunomide has been shown to inhibit viruses of the Herpesviridae family *in vitro*.

U.S. Patent No. 4,965,276 describes the use of leflunomide to treat chronic graft versus host disease and other autoimmune diseases such as systemic lupus erythematosus (SLE). Leflunomide has also been shown to exhibit antineoplastic activity against certain tumors (Xu X et al., *Biochem. Pharmacol.* 1999; 58:1405) and may act by inhibiting tumor neoangiogenesis (Waldman WJ et al., *Transplantation* 2001; 72:1578).

Despite the reported therapeutic benefits of leflunomide in the treatment and prevention of these disease processes, it has also been noted that administration of leflunomide may produce dose-limiting toxicity. Toxicity associated with high doses of leflunomide include anemia, diarrhea, and pathological changes of the small intestine and liver. In a study of the anti-cancer effects of leflunomide (inhibition of the oncogene product PDGF and PDGFr) observable beneficial effects were reported but the doses required for these effects produced unacceptable incidence of side effects, including severe weight loss, anorexia and anemia. (Ko, Yoo-Joung, et al. *Clinical Cancer Research*, 2001;7: 800-805)

Recently, it has been suggested that uridine therapy reduces the toxicity of leflunomide without significantly impairing the control of allograft rejection afforded by leflunomide. The utilization of exogenous uridine occurs through the pyrimidine salvage

pathway in the face of the leflunomide's blockade of the *de novo* pathway. Notwithstanding the potential benefits of administration of exogenous uridine, therapeutic use of uridine is complicated by its poor bioavailability (about 8% - 10%), requiring high dose administration for effective therapy. Moreover, high doses of uridine may cause gastrointestinal complications, including diarrhea, which are poorly tolerated in transplant patients dependent on intestinal function for therapeutic drug administration and which may exacerbate the diarrhea already caused by the leflunomide.

The present invention relates to the surprising discovery that the use of orotic acid alleviates the toxicity typically observed with leflunomide administration. Orotic acid (also known as vitamin B_{13}), an intermediate in the uridine synthetic pathway, appears to eliminate the pyrimidine deficiency caused by the malononitrilamides, metabolites (analogues of the active metabolite) of leflunomide, while avoiding the problems associated with uridine administration.

Accordingly, the invention provides methods and compositions useful in alleviating or reducing toxicity associated with leflunomide administration without reducing its bioactivity, e.g., without reducing its immunosuppressive activity. The present invention uses a bioavailable pyrimidine compound to ameloriate the toxic effects (e.g., anemia, diarrhea, heptotoxicity) caused by leflunomide compounds. As a result, high doses of leflunomide compounds can be administered with minimal danger of toxicity, all the while maintaining the therapeutic efficacy of the leflunomide compound. Co-administration of a leflunomide compound with orally bioavailable pyrimidines, such as orotic acid, provides for treatment opportunities using leflunomide compounds previously believed to be toxic, e.g., the present invention provides methods of reducing the toxicity of A77 1726 (a metabolite of leflunomide) analogs (described hereinbelow) by co-administering a leflunomide compound and, e.g., orotic acid. In addition to orotic acid, it is contemplated that additional analogs and metabolites of orotic acid or other bioavailable pyrimidine compounds may be suitable.

In one aspect, the invention provides pharmaceutical compositions particularly for oral administration. Such pharmaceutical compositions suitably include a leflunomide compound, a bioavailable, especially an orally bioavailable, pyrimidine compound or a salt thereof, and a pharmaceutically acceptable carrier.

In another aspect, the invention provides a method of extending the dosage range of a leflunomide compound. The method involves co-administering to a subject, e.g., a mammal, an effective dose of a leflunomide compound and an orally bioavailable

pyrimidine compound or salt thereof, e.g., orotic acid. Thus, the invention provides a method of administering high doses of a leflunomide compound without developing, i.e., reducing, toxicity resulting from leflunomide administration, which method comprises administering to a mammal, e.g., a human, treated with a leflunomide compound an effective amount of a bioavailable pyrimidine compound.

The invention further provides methods of prevention or treatment of certain disease states or processes that are suitably treated with a leflunomide compound. Such disease states or conditions include transplant rejection.

The invention will now be described in detail, those skilled in the art will appreciate that such a description of the invention is meant to be exemplary only and should not be viewed as limitative of the full scope thereof.

The following definitions used in the art may be useful in aiding the skilled practitioner in understanding the invention.

"Ameliorating" means observably reducing, alleviating, inhibiting or diminishing any undesirable effect or symptom of a condition or process associated with a disease state or any undesirable effect of a treatment of a disease state. For example, "amelioration of the effects of pyrimidine biosynthesis inhibition" may refer to any observable reduction in side effects caused by pyrimidine biosynthesis inhibition. Suitably, at least a 50% reduction in symptoms or side effects may be observed.

The term "co-administration" includes administration of two or more agents in a single unitary dosage form, administration of agents concurrently, and administration of agents sequentially, as long as they are given in a manner sufficient to allow both agents to achieve effective concentrations in the body. The agents may be in an admixture, as, for example, in a single tablet, or simply given concurrently. The agents may also be administered by different routes, e.g., one agent may be administered intravenously while the second agent is administered orally. In sequential administration, one agent may directly follow administration of the other or the agents may be administered episodically, i.e., one can be given at one time followed by the other at a later time.

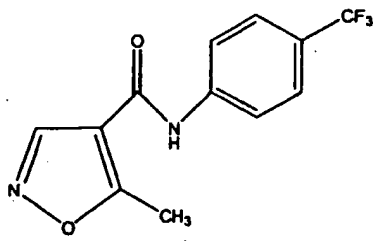
An "effective amount" of a compound, as used herein, means that amount of the compound or composition administered to a subject which is effective to produce its intended function, e.g., in one embodiment of the invention, prevention of transplant rejection. Thus, a "therapeutically effective amount" is an amount effective to produce therapeutic results. A "toxicity-reducing effective amount" is an amount effective to reduce toxicity. Typically, administration of effective amounts to a subject results in

observable amelioration of undesirable effects or symptoms of the condition or disease process which the subject is being treated.

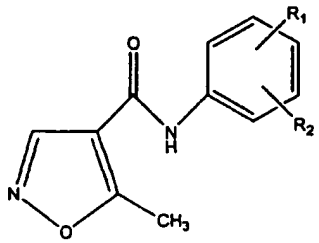
"Extending a dosage range" refers to providing a means by which greater doses of an agent may be administered to a subject to increase therapeutic effectiveness. Typically, extending a dosage range is useful, e.g., when efficacy of an agent is dose dependent but increased doses of the agent also leads to dose dependent toxicity. Alternatively, the term "extending a dosage range" may refer to administering agents believed to be toxic at any dosage.

A "leflunomide compound" refers generally to leflunomide, its analogs, its metabolites and analogs thereof.

Leflunomide is an isoxazole derivative with a chemical name of N-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxamide having the following chemical formula (I):



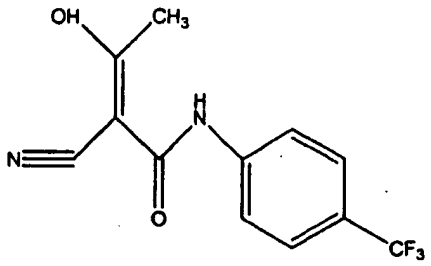
Analogs of leflunomide which may be useful in the practice of the methods of the invention may be represented by formula (II):



(I) (II)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, -NH-CO-CH_2Cl and NH-CO-CH_2Br. (See, e.g., U.S. Patent Nos. 4,087,535; 6,133,301; and 6,727,272)

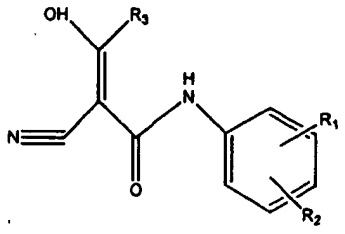
Leflunomide's active metabolite is referred to as "A77 1726" (2 cyano-3hydroxy-N-(4-trifluromethylphenyl)-buteneamide). After administration, leflunomide is rapidly converted to its active open-ring form, A77 1726, and is shown herein as formula (III):



(III)

This compound, a member of the malononitrilamide class of compounds, appears to account for leflunomide's activity and toxicity. Although its mechanism of action is not completely understood and wishing not be bound of any particular theory, A77 1726 is believed to exhibit at least two biochemical activities *in vivo*: inhibition of dihydroorotic acid dehydrogenase (DHODH) in the *de novo* synthesis of pyrimidine nucleotide triphosphates; and inhibition of selected tyrosine kinases involved in T-cell, B-cell, vascular smooth muscle cell, endothelial cell, fibroblast and tumor cell signaling cascades. A77 1726 also has been reported to block NFkB and AP-1 activation in peripheral blood lymphocytes *in vitro*. Additional mechanisms remain to be discovered.

Suitable malononitrilamide compounds which are analogs of A77 1726 may be useful in the practice of the methods of the invention and may be represented by formula (IV):



(IV)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, $-NH-CO-CH_2Cl$ and $NH-CO-CH_2Br$ and wherein R_3 is selected from the group consisting of C_{1-5} alkyl, C_{2-5} alkenyl, C_{2-5} alkynyl, and C_{3-6} cycloalkyl. Compounds of formula (IV) include FK7778 and FK779 wherein R_1 is -H, R_2

is $-CF_3$ and R_3 is butynyl (i.e., 2-cyano- 3-hydroxy-N-[4-(lufluaromethyl) phenyl]-2-hepten-6-ynoic acid amide) and R_1 is -H, R_2 is cyano and R_3 is cyclopropyl (i.e., 2-cyano-3-hydroxy-3-cyclopropyl-N-(4-cyanophenyl)-propenic acid amide), respectively.

In some embodiments of the methods of the invention, the leflunomide compound is administered as a prodrug to subjects and subsequently converted *in vivo* to its active malononitrilamide compound, defined above. It is contemplated, however, that the malononitrilamide compound may also be directly administered, and the term "leflunomide compound", as defined above, also refers to malononitrilamide compounds. It is to be understood that discussion herein regarding leflunomide compound administration is meant to be inclusive of malononitrilamide compound administration, as appropriate.

Leflunomide and its analogs can be prepared by known methods such as those described in U.S. Patent No. 6,723,855; U.S. Patent No. 6,727,272; U.S. Patent No. 6,133,301; U.S. Patent No. 5,905,090; U.S. Patent No. 4,087,535; U.S. Patent No. 4,351,841; and U.S. Patent No. 4,965,276, all of which are incorporated herein by reference in their entireties. Leflunomide is also commercially available from chemical suppliers, such as SynQuest Corp. (Chicago, Illinois).

As used herein, "bioavailable" in reference to a pyrimidine compound is one that is at least about 20% bioavailable after administration. "Orally bioavailable" in reference to a pyrimidine compound is a compound that is at least about 20% bioavailable after oral administration.

The phrase "pharmaceutically acceptable carrier," as used herein, means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject chemical from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and

polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations. Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

As used herein, "preventing," "reducing risk" or "reduced risk" as it applies to a particular condition or disease process, refers to observable results which tend to demonstrate that a particular treatment or treatment regimen has resulted in a significant decrease in incidence of the condition or disease process in a treated population, as compared to an untreated or control population. Suitably, risk is reduced, or a condition is prevented, if at least 50% of the treated population are not afflicted.

As used herein, a "pyrimidine compound" refers to a compound that is bioavailable, especially orally bioavailable, and useful either directly or as intermediates in pathways for supplying pyrimidine nucleotides. A suitable pyrimidine compound is, e.g., orotic acid. Other suitable pyrimidine compounds include orotic acid salts, triacetyl uridine and salts thereof, cytidine, acylated cytidine and salts thereof.

It is to be understood that the phrase "a salt thereof," when used herein to refer to pharmaceutical compositions, means physiologically compatible salts which are pharmaceutically acceptable. Examples of suitable salts are alkali metal (e.g., sodium), alkaline earth metal (e.g., calcium, magnesium) and ammonium salts, including those of physiologically tolerated organic ammonium bases.

As used herein, the term "treating" means observably reducing any undesirable effect or symptom of a condition or process associated with a disease state or any undesirable effect of a treatment of a disease state. Suitably, at least a 50% reduction in symptoms or side effects may be observed in a treated subject.

It also is specifically understood that any numerical value recited herein includes all values from the lower value to the upper value, i.e., all possible combinations of numerical values between the lowest value and the highest value enumerated are to be considered to be expressly stated in this application. For example, if a concentration range is stated as 1% to 50%, it is intended that values such as 2% to 40%, 10% to 30%, or 1%

to 3%, etc., are expressly enumerated in this specification. These are only examples of what is specifically intended.

In one embodiment, the present invention provides an effective method for reducing the risk of toxicity of leflunomide compounds used for the treatment of transplant rejection. Particularly, the present invention relates to therapeutic methods for ameliorating the risk of toxic side effects of a leflunomide compound, and thus permitting extending dosing of such compounds. The present invention provides treatment of a patient suffering from the toxic side effects of a leflunomide compound with an orally bioavailable pyrimidine compound based on a novel treatment protocol. The pyrimidine compound is suitably orotic acid, a salt thereof (e.g., sodium orotate), or a triacetyluridine. The pyrimidine compound is provided to the patient to significantly reduce the toxic effects of a leflunomide compound, e.g., anemia and diarrhea resulting in reduced hematocrit and weight loss. These attributes are achieved through specific properties of the pyrimidine compounds and the novel treatment protocol as described herein.

A suitable pyrimidine compound is orotic acid. Orotic acid is found in small concentrations in the blood of healthy individuals. Elevated levels appear to be free of any appreciable complications in humans and animals. Several conditions are known, however, in which orotic acid levels in the blood are elevated, e.g., in urea synthesis defects, in individual Hereditary Oroticaciduria treated with uridine for years, and in patients receiving allopurinol, without recognized specific damage. In addition, it is recognized that blood levels of orotic acid are elevated several fold in renal failure without specifically recognized toxicity.

Orotic acid may be prepared by condensation of urea with the monoethyl ester of oxalacetic acid in methanol. Other preparation methods, including those utilizing biotechnological methods known in the art, are also suitable. Orotic acid may be administered in its free acid form, or may be administered as a pharmaceutically acceptable salt. Examples of suitable salts are alkali metal (e.g., sodium orotate), alkaline earth metal (e.g., magnesium orotate or calcium orotate) and ammonium salts, including those of physiologically tolerated organic ammonium bases. Orotic acid is also commercially available from chemical suppliers, such as Aldrich (Milwaukee, Wisconsin).

Also included among the bioavailable pyrimidine compounds of the invention are those comprising certain known acyl derivatives of uridine, i.e., acylated uridines, e.g., 2', 3', 5'-tri-O-acetyl uridine (or triacetyluridine (TAU)), 2', 3', 5'-tri-O-propionyl uridine, or 2', 3', 5'-tri-O-butyryl uridine. TAU, for example, is orally bioavailable. TAU and other

acyl derivatives of uridine can be made by methods known in the art (see, e.g., U.S. Patent No. 6,316,426; U.S. Published Patent Application 2002/0035086 and references cited therein, all of which are incorporated herein by reference); TAU is also commercially available through SP-Chemicals, Ludwigshofen, DK.

The pyrimidine compounds of the invention also include cytidine and certain acyl derivatives of cytidine, i.e., acylated cytidines, e.g., 2', 3', 5'-tri-O-acetyl cytidine (or triacetylcytidine or TAC), 2', 3', 5'-tri-O-propionyl cytidine, or 2', 3', 5'-tri-C-butyryl cytidine. TAC and other acyl derivatives of cytidine can be made by methods known in the art (see, e.g., U.S. Published Patent Application 2002/0035086 and references cited therein, all of which are incorporated herein by reference).

Suitably, the pyrimidine compound may be administered in an amount that is approximately that which is needed to provide the daily pyrimidine synthesis requirements minus what is provided through the salvage pathway. The total pyrimidine synthesis in adult humans is estimated to be from about 4 mmol/day to about 12 mmol/day, or about 450 to about 700 mg of uridine per day. (Bono VH, Weissman SM, Frei E. The effect of 6-azauridine administration on de novo pyrimidine production in chronic myelogenous leukemia. *J Clin Invest* 1964; 43:1486; Smith LH Jr. Pyrimidine Metabolism in Man. *New Engl J of Med* 1973; 288:764-772.) For orotic acid, this would amount to approximately 1000 mg per day. It is not believed to be necessary, however, to provide the entire daily supply of pyrimidine since the salvage pathway provides some of the total. It is believed that the bioavailability of orotic acid is approximately 50%. Therefore, for oral administration in an adult, an effective amount of orotic acid would be about 500 mg to about 2,000 mg per day. A similar dosing is contemplated for TAU.

For patients being treated with a leflunomide compound, the targeted blood level of active metabolite (A77 1726) is suitably between about 50 μg/mL and about 100 μg/mL. The maintenance dose may be adjusted by one of ordinary skill in the art to attain the desired blood level range of active metabolite. If pyrimidine deficiency is prevented with co-administration of a pyrimidine compound, the targeted blood level of active metabolite may be substantially higher, e.g. about 200 μg/mL or about 600 μM.

Mammalian transplant recipients, such as kidney recipients and bone marrow recipients, may be suitably treated in accordance with the present invention. Typically, a human transplant recipient is administered a leflunomide compound at a dose of about 100 mg per day for five days, and then 40 mg per day thereafter as a maintenance dose. The co-administration of the leflunomide compound and a pyrimidine compound will extend

the therapeutic dose of the leflunomide compound to more than 200 mg/patient/day. This method will prevent the development of or reduce the risk of toxicity (e.g., anemia, diarrhea, hepatotoxicity) and will result in achieving concentrations of the leflunomide compounds that can suppress rejection. It is expected that the use of this methodology will allow up to 10-fold or higher increase in dosage level of leflunomide compounds with minimal danger of developing toxicity to the patient. In other words, the present invention provides a method of administering a toxic dose of a leflunomide compound by administering an effective amount of a pyrimidine compound. By "toxic dose" or "high dose" is meant a dose of the leflunomide compound which when administered to a mammal such as a human often results in the toxic effects, e.g., anemic and diarrhea as well as other pathological changes. In humans, a high dose may be more than 200 mg per day.

In administration of leflunomide compounds, toxicity-reducing effective amounts of the bioavailable pyrimidine compounds are co-administered to subjects with allografts or xenografts, thereby ameloriating the toxic effects of the leflunomide compounds, i.e., weight gain is promoted and hematocrit maintained, with significantly less risk of toxicity than is observed after the same amount of leflunomide compound alone is administered. The risk of toxicity, associated with the administration of high doses of leflunomide compounds, is lowered by co-administering the leflunomide with a pyrimidine compound, especially an orally bioavailable pyrimidine compound. Thus, the combination therapy for use in accordance with the present invention provides an improved therapeutic index relative to leflunomide compounds alone given in conventional protocols. The treatment protocol in accordance with the present invention provides reduced risk of toxicity, (e.g., improved weight gain and hematocrit) i.e., little or no clinical symptoms or signs of toxicity.

The pyrimidine compounds of the present invention given in the illustrated dosing regimen, thus, overcome the toxicities of leflunomide compounds and can be considered beneficial agents for the control and treatment of toxicity associated with treatment with leflunomide compounds. In such combination therapy, the leflunomide compound may be co-administered with the pyrimidine compound concurrently, sequentially, or in a unitary formulation. For efficiency, ease of administration and patient compliance, the latter is especially suitable.

A pharmaceutical composition of a leflunomide compound and a bioavailable pyrimidine compound is suitably formulated in unit dosage form of about 500 mg to about

2000 mg of pyrimidine compound and about 20 mg to about 100 mg of leflunomide compound. Lower doses of pyrimidine compound may be adequate for children or individuals with reduced clearance of pyrimidines, such as individuals with reduced kidney function or other conditions that might reduce pyrimidine elimination.

The dosage form of compositions of the invention is not particularly limited, and any form suitable for oral administration may be used in accordance with standard formulation procedures known in the art. Examples of dosage forms suitable for oral administration include, but are not limited to, solid formulations and aqueous formulations. Solid formulations suitable for oral administration include capsules, tablets, powders or granules, and may include excipients such as lactose, glucose, sucrose or mannitol; a disintegrator such as starch or sodium alginate; a lubricant such as magnesium stearate or talc; a binder such as polyvinyl alcohol, hydroxypropylcellulose or gelatin; a surfactant such as fatty acid ester; and a plasticizer such as glycerine, and the like. Aqueous formulations suitable for oral administration include solutions, emulsions, syrups and suspensions. Such formulations may also include sugars such as sucrose, sorbitol or fructose; glycols such as polyethylene glycol or propylene glycol, oils such as sesame oil, olive oil or soybean oil, antiseptics such as p-hydroxybenzoate, and flavors such as strawberry and peppermint.

While, perhaps, less convenient than an oral formulation, it is also contemplated that the compositions may be formulated for rectal administration in accordance with standard formulations procedures known in the art. Examples of dosage forms suitable for rectal administration include solid suppositories, mucoadhesive suppositories, solutions, suspensions, retention enemas, gels, forms and ointments.

It is further contemplated that a dosage form of the compositions in accordance with the present invention may be formulated for immediate release, delayed release or controlled release. Many controlled release systems are known in the art (see e.g., U.S. Patent 5,529,991). Sustained, controlled or directed release compositions can be formulated, e.g., in liposomes, via laser originated openings or those wherein the active compound is protected with differentially degradable coatings, such as by microencapsulation, multiple coatings, etc.

For example, in diffusional systems, the release rate of drugs is affected by their rate of diffusion through a water-insoluble polymer. There are generally two types of diffusional systems, formulations in which a core of drug is surrounded by polymeric membrane; and matrix devices in which dissolved or dispersed drug is distributed

substantially uniformly and throughout an inert polymeric matrix. In actual practice, many systems that utilize diffusion can also rely to some extent on dissolution to determine the release rate.

Common materials used as the membrane barrier coat, alone or in combination, include but are not limited to, hardened gelatin, methyl and ethyl-cellulose, polyhydroxymethacrylare, polyvinylacetate, and various waxes.

In matrix systems, three major types of material are frequently used in the preparation of the matrix systems which include insoluble plastics, hydrophilic polymers, and fatty compounds. Plastic matrices which have been employed include methyl acrylate-methyl methacrylate, polyvinyl chloride and polyethylene. Hydrophilic polymers include methyl cellulose, hydroxypropylcellulose, hydroxpropyl-ethylcellulose, and its derivatives and sodium carboxy-methylcellulose. Fatty compounds include various waxes such as carnauba wax, and glyceryl tristearate. These matrix systems are prepared by methods well known to those skilled in the art. These methods of preparation generally comprise mixing the drug with the matrix material and compressing the mixture into a suitable pharmaceutical layer. With wax matrices, the drug is generally dispersed in molten wax, which is then congealed, granulated and compressed into cores.

The most common method of microencapsulation is coacervation, which involves addition of a hydrophilic substance to a colloidal dispersion. The hydrophilic substance, which operates as the coating material, is selected from a wide variety of natural and synthetic polymers including shellacs, waxes, starches, cellulose acetates, phthalate or butyrate, polyvinyl-pyrrolidone, and polyvinyl chloride. After the coating material dissolves, the drug inside the microencapsule is immediately available for dissolution and absorption. Drug release, therefore, can be controlled by adjusting the thickness and dissolution rate of the coat. For example, the thickness can be varied from less than one μm to 200 μm by changing the amount of coating material from about 3 to 30 percent by weight of the total weight. By employing different thicknesses, typically three of four, the active agent will be released at different, predetermined times to afford a delayed release effect.

Approaches to further reducing the dissolution rate include, for example, coating the drug with a slowly dissolving material, or incorporating the drug into a formulation with a slowly dissolving carrier. Thus, encapsulated dissolution systems are prepared either by coating particles or granules of drug with varying thickness or slowly soluble polymers or by microencapsulation.

While it is contemplated that a unitary oral formulation containing both a leflunomide compound and a bioavailable pyrimidine compound provides ease of administration and patient compliance, it is also understood that the compounds may be administered separately but packaged together, e.g., in a blister pack, with instructions for administration.

Although examples of suitable dosage ranges are provided, it will be appreciated that the specific dosages administered in any given case will be adjusted in accordance with the specific compounds being administered, the disease to be treated, the condition of the subject and other relevant medical factors that may modify the activity of leflunomide, the response of the subject or the amount of bioavailable pyrimidine compound needed, as is well known by those skilled in the art. For example, the specific dose for a particular patient depends on age, body weight, general state of health, diet, the timing and mode of administration, the rate of excretion, and medicaments used in combination and the severity of the particular disorder to which the therapy is applied. Dosages for a given patient can be determined using conventional considerations, e.g., by customary comparison of the differential activities of the subject compounds and of a known agent, such as by means of an appropriate conventional pharmacological protocol.

The following examples are provide to assist in a further understanding of the invention. The particular materials and conditions employed are intended to be further illustrative of the invention and are not limiting upon the reasonable scope thereof.

Example 1: Effect of orotic acid administration on efficacy of leflunomide in the treatment of acute rejection

Lewis Rats which received heart transplants from Brown-Norway rats were observed for graft survival and inflammation (scored on a 0-3 scale, with 0 being no inflammation). Treatments included 0, 5, 10 or 15 mg/kg of leflunomide in combination with 0 or 100 mg/kg orotic acid. The results are tabulated below.

Number animals	Dose leflunomide (mg/kg/day)	Dose orotic acid (mg/kg/day)	Graft survival (days)	Inflammation score(Mean) (0-3)
8	0	0	6.9	3.0
8	5	0	>30	2.0
5	5	100	>30	2.3
5	10	0	>30	1.7
5	10	100	>30	1.8
5	15	0	>30	1.5
5	15	100	>30	1.6

Administration of leflunomide reduced the intensity of the rejection reaction, as shown by the inflammation score, in a dose-related fashion. Orotic acid did not significantly affect the efficacy of leflunomide to reduce the intensity of the rejection reaction.

Example 2: Effect of orotic acid on leflunomide toxicity as measured by changes in body weight

As noted previously, the most observed symptoms of experimental leflunomide-induced toxicity are anemia and diarrhea resulting in weight loss or reduced weight gain. Lewis rats with either an allograft or xenograft weighing between 200 and 235 grams were divided into four treatment groups. Each group received 30 mg/kg/day of leflunomide, a high, toxic dose: Group I received leflunomide only; Group II received leflunomide plus 36 mg/kg/day of sodium orotate by gavage; Group III received leflunomide plus 100 mg/kg/day of orotic acid by gavage; and Group IV received 250 mg/kg/day of uridine by IP injection. Weight of each rat was measured at week 1 and week 4 post commencement of therapy. The results are tabulated below.

GROUP I (leflunomide only)

Treatment-dosage (mg/kg/day)	Weight at Week 1 Grams	Weight at Week 4 grams	Change in body weight
L30	234	246	13
L30	211	236	25
L30	241	237	-4
L 30	253	264	11
L 30	246	255	9
L 30	203	253	50
			Mean: 20.8

B-40

GROUP II

Treatment-dosage (mg/kg/day)	Weight at Week 1 Grams	Weight at Week 4 grams	Change in body weight
L30 + O36	270	266	-4
L30 + O36	244	235	-9
L30 + O36	250	218	-32
L30 + O36	205	267	62
L30 + O36	198	240	42
L30 + O36	211	262	51
			Mean: 18.3

GROUP III

Treatment-dosage (mg/kg/day)	Weight at Week 1 Grams	Weight at Week 4 grams	Change in body weight
L30 + O100	210	250	40
L30 + O100	208	265	57
L30 + O100	209	250	41
L30 + O100	207	271	66
L30 + O100	210	268	58
L30 + O100	197	258	61
			Mean: 53.7

GROUP IV

Treatment-dosage (mg/kg/day)	Weight at Week 1 Grams	Weight at Week 4 grams	Change in body weight
L30 + U250	207	231	24
L30 + U250	204	242	38
L30 + U250	208	230	22
L30 + U250	197	241	44
L30 + U250	204	272	68
L30 + U250	211	275	64
			Mean: 43.3

* L30 refers to administration of 30 mg/kg per day of leflunomide; O36 refers to 36 mg/kg per day of orotic acid; O100 refers to 100 mg/kg per day of orotic acid; and U250 refers to 250 mg/kg per day of uridine given IP.

The results showed that the use of a combination of leflunomide and orotic acid or a salt thereof significantly improved weight gain compared to use of leflunomide alone.

Example 3: Effect of orotic acid on leflunomide toxicity as measured by hematocrit

The experiment of Example 2 was repeated in Lewis rats and the hematocrit measured weekly for four weeks. The rats receiving treatment were divided into five groups wherein each group received 30/mg/kg/day leflunomide, a toxic, high dose. Group

I received leflunomide only. Group II received the leflunomide dose plus 36/mg/kg/day of sodium orotate; group III received the leflunomide dose plus 100mg/kg/day of sodium orotate; group IV received the leflunomide dose plus 88mg/kg/day of orotic acid; and group V received the leflunomide dose plus 250mg/kg/day of uridine given IP. A baseline hematocrit was measured, and hematocrits of each rat were measured at weeks 1-4 post commencement of therapy. The results are tabulated below.

GROUP I

Treatment-dosage (mg/kg/day)	Hct Week 0	Hct Week 1	Hct Week 2	Hct Week 3	Hct Week 4
L30	51	47	39	31	17
L30	57	52	46	33	20
L 30	54	55	54	37	30
L 30	55	49	48	33	24
L 30	52	50	46	37	22
L 30	53	53	44	41	30
					Mean: 23.8

GROUP II

Treatment-dosage (mg/kg/day)	Hct Week 0	Hct Week 1	Hct Week 2	Hct Week 3	Hct Week 4
L30 + O36	53	50	48	39	20
L30 + O36	57	50	49	32	14
L30 + O36	53	51	39	27	10
L30 + O36	54	52	49	47	44
L30 + O36	51	53	40	36	35
L30 + O36	52	49	44	30	20
					Mean: 23.8

GROUP III

Treatment-dosage (mg/kg/day)	Hct Week 0	Hct Week 1	Hct Week 2	Hct Week 3	Hct Week 4
L30 + O100	53	48	43	41	32
L30 + O100	52	49	51	46	44
L30 + O100	51	46	44	42	45
L30 + O100	57	53	50	41	33
L30 + O100	51	49	46	34	22
L30 + O100	52	51	53	43	46
					Mean: 37

GROUP IV

Treatment-dosage (mg/kg/day)	Hct Week 0	Hct Week 1	Hct Week 2	Hct Week 3	Hct Week 4
L30 + U250	52	47	48	50	46
L30 + U250	56	50	51	51	49
L30 + U250	54	46	43	41	25
L30 + U250	51	51	48	38	24
L30 + U250	53	49	54	51	44
L30 + U250	54	54	49	43	38
					Mean: 37.7

GROUP V

Treatment-dosage (mg/kg/day)	Hct Week 0	Hct Week 1	Hct Week 2	Hct Week 3	Hct Week 4
L30+ OA88	56	50	53	52	50
L30+ OA88	54	51	54	52	54
L30+ OA88	52	49	44	38	32
					Mean: 45.3

The results demonstrated that use of the combination of leflunomide and orotic acid or sodium orotate, provided significantly higher hematocrits than in the use of leflunomide alone.

As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to a composition containing "a pyrimidine compound" includes a mixture of two or more pyrimidine compounds. It should also be noted that the term "or" is generally employed in its sense including "and/or" unless the content clearly dictates otherwise.

All publications, patents and patent applications referenced in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications, patents and patent applications are herein expressly incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference. In case of conflict between the present disclosure and the incorporated patents, publications and references, the present disclosure should control.

The invention has been described with reference to various specific embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

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We claim:

1. A pharmaceutical composition in unit dosage form for oral administration comprising an effective amount of a leflunomide compound; and an orally bioavailable pyrimidine compound, salts thereof or a combination thereof; together in a pharmaceutically acceptable carrier.

2. The composition of claim 1 wherein the pyrimidine compound is orotic acid, a salt thereof, triacetyluridine, a salt thereof, cytidine, a salt thereof, an acylated cytidine, a salt thereof, or a combination thereof.

3. The composition of claim 1, wherein the unit dosage contains 500 mg to 2000 mg of pyrimidine compound.

4. The composition of claim 1, wherein the leflunomide compound is leflunomide, A771726 or FK778.

5. The composition of claim 1, wherein the composition is formulated for controlled release.

6. The composition of claim 1, wherein the composition is formulated for rectal administration.

7. A pharmaceutical composition comprising a formulation for oral administration, the formulation comprising a therapeutically effective amount of leflunomide, and orotic acid or a salt thereof, and a pharmaceutically acceptable carrier.

8. A method of reducing toxicity associated with administration of a leflunomide compound to a patient in need thereof, comprising administering to the patient a toxicity-reducing amount of a bioavailable pyrimidine compound.

9. The method of claim 8, wherein the pyrimidine compound is orotic acid, a salt thereof, triacetyaluridine, a salt thereof, cytidine, a salt thereof, an acylated cytidine, a salt thereof, or a combination thereof.

10. The method of claim 8, wherein the pyrimidine compound is administered orally.

11. The method of claim 8, wherein the pyrimidine compound is administered in a daily dosage of from about 500 mg to about 2000 mg.

12. The method of claim 8, wherein the pyrimidine compound is co-administered substantially simultaneously with the leflunomide compound.

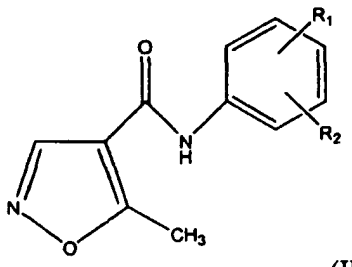
13. The method of claim 8, wherein the patient is a recipient of a transplant.

14. The method of claim 13, wherein the transplant is an allograft or a xenograft.

15. The method of claim 13, wherein the transplant is a heart, a kidney or bone marrow.

16. The method of claim 8, wherein the leflunomide compound is selected from a compound having

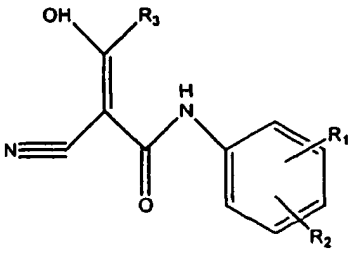
a) formula (II):



(II)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, $-NH-CO-CH_2Cl$ and $-NH-CO-CH_2Br$;

or formula (IV):



(IV)

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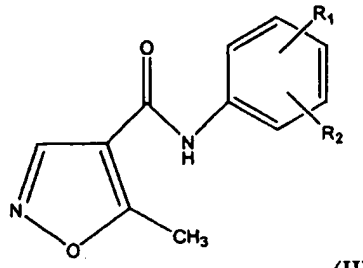
wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, $-NH-CO-CH_2Cl$ and $-NH-CO-CH_2Br$, and R3 is selected from the group consisting of C_{1-5} alkyl, C_{2-5} alkenyl, C_{2-5} alkynyl, and C_{3-6} cycloalkyl.

17. A method of extending the dosage range of a leflunomide compound comprising co-administering to a subject:

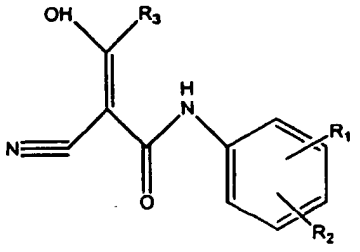
a) an effective amount of a leflunomide compound of formula (II):



(II)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, - $-NH-CO-CH_2Cl$ and $-NH-CO-CH_2Br$;

or formula (IV):



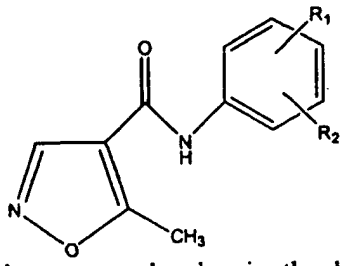
(IV)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, $-NH-CO-CH_2Cl$ and $-NH-CO-CH_2Br$; and R_3 is selected from the group consisting of C_{1-5} alkyl, C_{2-5} alkenyl, C_{2-5} alkynyl, and C_{3-6} cycloalkyl; and

b) a toxicity-reducing amount of an orally bioavailable pyrimidine compound selected from the group consisting of orotic acid, a salt thereof, triacetyl uridine, a salt thereo, cytidine, a salt thereof, an acylated cytidine, a salt thereof, and a combination thereof.

18. A method of administering a toxic dose of a leflunomide compound to a mammal, comprising administering to the mammal an amount of an orally bioavailable pyrimidine compound sufficient to reduce the toxic effects of the leflunomide compound.

19. A method of reducing toxicity associated with the administration of a therapeutically effective amount of a leflunomide compound to a mammal, comprising: orally administering to the mammal a bioavailable pyrimidine compound selected from orotic acid, a salt thereof, triacetyluridine, , a salt thereof, cytidine, a salt thereof, an acylated cytidine, a salt thereof, and a combination thereof, in an amount effective to reduce the toxicity without blocking therapeutic

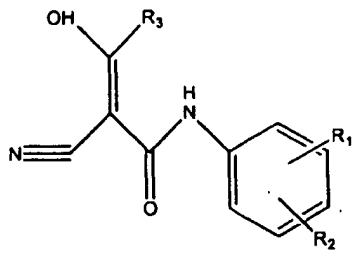


effect of the leflunomide compound, wherein the leflunomide compound is a compound of formula (II)

or formula (IV).

20. The method of claim 19 wherein the pyrimidine compound is orotic acid or a salt thereof.

21. A method of treating rejection in a transplant recipient comprising co-



administering a therapeutically effective amount of a leflunomide compound and a toxicity-reducing effective amount of bio-available pyrimidine compound.

22. The method of claim 21, wherein the pyrimidine compound is orally bio-available.

23. The method of claim 22, wherein the pyrimidine compound is orotic acid, a salt thereof, triacetyl uridine, a salt thereof, cytidine, a salt thereof, an acylated cytidine, a salt thereof, or a combination thereof.

24. A method of achieving an effect in a patient comprising co-administering an effective amount of a leflunomide compound and an effective amount of orotic acid, a salt thereof, triacetyl uridine, a salt thereof, or a combination thereof, wherein the effect is treatment of rejection of a transplant, wherein the transplant is heart, kidney or bone marrow.

25. A pharmaceutical combination comprising a packaging having a plurality containers, at least one container containing a leflunomide compound, at least one other container containing a bioavailable pyrimidine compound, and an instructions for co-administering the leflunomide compound and the pyrimidine compound to a subject who is a transplant recipient.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/26145

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 31/42
US CL : 514/378
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/378

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6,133,301 A (BARTLETT) 17 October 2000(17.10.2000), see entire document.	1-25

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search	Date of mailing of the international search report
26 November 2005 (26.11.2005)	08 DEC 2005
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized officer Michael Hartley Telephone No. (571) 272.1600

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Biochemical Pharmacology, Vol. 51, pp. 1601–1611, 1996.

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in mice

5-(*m*-Benzyloxybenzyl)barbituric Acid Acyclonucleoside, a Uridine Phosphorylase Inhibitor, and 2′,3′,5′-Tri-O-Acetyluridine, a Prodrug of Uridine, as Modulators of Plasma Uridine Concentration

IMPLICATIONS FOR CHEMOTHERAPY

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ABSTRACT. 5-(*m*-Benzyloxybenzyl)barbituric acid acyclonucleoside (BBBA), the most potent inhibitor known of uridine phosphorylase (UrdPase, EC 2.4.2.3), the enzyme responsible for uridine catabolism, and 2′,3′,5′-tri-O-acetyluridine (TAU), a prodrug of uridine, were used to investigate the possibility of improving the bioavailability of oral uridine in mice. Oral BBBA administered at 30, 60, 120, and 240 mg/kg increased the concentration of plasma uridine (2.6 ± 0.7 μM) by 3.2-, 4.6-, 5.4-, and 7.2-fold, respectively. After administration of 120 and 240 mg/kg BBBA, plasma uridine concentration remained 3- and 6-fold, respectively, higher than the plasma concentration at zero time (C_0) for over 8 hr. On the other hand, BBBA did not change the concentration of plasma uracil. TAU was far more superior than uridine in improving the bioavailability of plasma uridine. The relative bioavailability of plasma uridine released from oral TAU (53%) was 7-fold higher than that (7.7%) obtained by oral uridine. Oral TAU at 460, 1000, and 2000 mg/kg achieved area under the curve (AUC) values of plasma uridine of 82, 288, and 754 μmol · hr/L, respectively. Coadministration of BBBA with uridine or TAU further improved the bioavailability of plasma uridine resulting from the administration of either alone and reduced the C_{max} and AUC of plasma uracil. Coadministration of BBBA at 30, 60, and 120 mg/kg improved the relative bioavailability of uridine released from 2000 mg/kg TAU (53%) by 1.7-, 2.7-, and 3.9-fold, respectively, while coadministration of the same doses of BBBA with an equimolar dose of uridine (1320 mg/kg) increased the relative bioavailability of oral uridine (7.7%) by 4.1-, 5.3-, and 7.8-fold, respectively. Moreover, the AUC and C_{max} of plasma uridine after BBBA (120 mg/kg) coadministration with TAU were 3.5- and 11.5-fold, respectively, higher than those obtained from coadministration of BBBA with an equimolar dose of uridine. The exceptional effectiveness of the BBBA plus TAU combination in elevating and sustaining high plasma uridine concentration can be useful in the management of medical disorders that are remedied by administration of uridine as well as to rescue or protect from host-toxicities of various chemotherapeutic pyrimidine analogues. BIOCHEM PHARMACOL 51;12:1601–1611, 1996.

KEY WORDS. uridine; phosphorylase; inhibitor; prodrug; chemotherapy

The pyrimidine nucleoside, uridine, has been used successfully as a "protective" and/or "rescuing" agent against host-toxicity of various anti-cancer (e.g. 5-fluorouracil) [1–4] and anti-AIDS (e.g. 3′-azido-3′-deoxythymidine and 2′,3′-dideoxycytidine) [5–7] drugs without interfering with their chemotherapeutic efficacy. The use of uridine as an adjunct in therapy is not limited to the treatment of cancer and AIDS. Uridine was shown to protect from the toxic effects of different anti-inflammatory and immunosuppressive agents used in the treatment of various auto-immune diseases and transplant rejection [8–11], and potentiate the anti-psychotic action of traditional neuroleptics [12, 13]. Uridine has also been used as a therapeutic agent in the treatment of several other medical disorders including: CNS disorders (e.g. cerebrovascular disorders and convulsions) [14–25], sleep promotion [26], muscle performance [27, 28], liver diseases [29–31], diabetic neuropathy [32], cardiac [illegible], and hereditary orotic aciduria [39]. However, because of its rapid clearance [40–48], it is necessary to administer substantial doses of uridine (10–12 g/m^2) [41] to attain and sustain the high plasma uridine concentrations (70 μM) [49] required to achieve the protective or rescuing effects. Unfortunately, such large doses of uridine also produce dose-limiting side-effects (e.g. phlebitis, pyrogenic reactions, and diarrhea) [42, 45, 50–52]. Therefore,

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alternative approaches to increase uridine bioavailability to the required concentrations must be sought.

Uridine is present in constant concentrations (1–5 μM) in the plasma of various species [53–57]. However, plasma uridine half-life is approximately 2 min [58]. Hence, the turnover of the plasma uridine must be rapid and efficient. Indeed, more than 90% of the circulating uridine is catabolized in a single pass through the liver by the activity of hepatic UrdPase† (EC 2.4.2.3), while constant amounts of uridine are synthesized *de novo* and released into the hepatic vein blood [40, 58]. Less than 2% of the uridine metabolized by the liver is salvaged and recovered in the uracil nucleotide pool in tissues of whole animals [55, 57, 59, 60], perfused rat liver [40, 58], and isolated liver cells [61]. The remainder is catabolized rapidly to products beyond uracil in the pyrimidine catabolic pathway [48, 62, 63].

One approach to maintain a high uridine concentration over a prolonged period is the use of UrdPase inhibitors to block the rapid catabolism of uridine to uracil. Inhibition of uridine catabolism by UrdPase inhibitors would lead to increased plasma uridine concentration as a result of the continuous *de novo* biosynthesis of uridine in the liver. Indeed, UrdPase inhibitors have been used to increase the concentration and half-life of plasma uridine [48, 49, 51, 62, 64–67] and the salvage or uridine by various tissues [49, 51, 68, 69].

Another approach to increase uridine bioavailability is to modify the structure of uridine to prevent its rapid catabolism by UrdPase and enhance its uptake into tissues where the modified uridine can be utilized. For this purpose, TAU (Fig. 1) has been designed and synthesized as a prodrug of uridine [70]. The acetyl groups of TAU increase the lipophilicity of uridine, thus enhancing its transport from the gastrointestinal tract to the blood stream and its reabsorption from the renal tubules, while rendering TAU resistant to catabolism by UrdPase [70]. Uridine is released from TAU by the action of plasma esterases. Furthermore, plasma has very little, if any, UrdPase activity; thus, the catabolism of uridine in plasma is minimal (unpublished data). This could eventually lead to a stable source for sustained delivery of high concentrations of uridine in plasma [70].

In the present study, we investigated the two approaches using BBBA (Fig. 1), the best known inhibitor of UrdPase [71–74], and/or TAU, as a prodrug of uridine, to improve the bioavailability and pharmacokinetics of plasma uridine in mice.

† *Abbreviations*: AUC, area under the curve; BBBA, 5-(*m*-benzyloxybenzyl)barbituric acid acyclonucleoside; C_0, plasma concentration at zero time; C_{max}, peak plasma concentration; Cl_T, total plasma clearance; DHUDase, dihydrouracil dehydrogenase, EC 1.3.1.2; HPMC, hydroxypropylmethylcellulose; *MRT*, mean residence time; $T_{1/2}$, elimination half-life; TAU, 2′,3′,5′-tri-O-acetyluridine; TCA, trichloroacetic acid; T_{max}, time to peak plasma concentration; UrdPase, uridine phosphorylase, EC 2.4.2.3; and $V_{d_{ss}}$, volume of distribution at steady state.

MATERIALS AND METHODS

Animals

Female CD-1 mice, 18–20 g, were purchased from Charles River Laboratories (Wilmington, MA) and housed 5/cage with food and water *ad lib.* under a normal light cycle (light, 6:00 a.m. to 6:00 p.m.; dark, 6:00 p.m. to 6:00 a.m.).

Chemicals

Uridine, tri-*n*-octylamine, freon (1,1,2-trichlorotrifluoroethane) and HPMC were purchased from the Sigma Chemical Co. (St. Louis, MO). Heparinized Natelson pipets, ammonium acetate, acetonitrile (HPLC grade), TCA, Gelman Acrodisc LC 13 PVDF 0.2 μm filters, and ethyl ether (anesthetic grade) were purchased from Fisher Scientific (Pittsburgh, PA). [6-^{14}C]Uracil (55 Ci/mol) and [2-^{14}C]uridine (56 Ci/mol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). TAU was provided by Dr. Reid von Borstel, Pro-Neuron, Inc. (Rockfield, MD). BBBA was synthesized as described previously [71, 72].

Administration of Drugs

For oral administration, uridine (alone or with BBBA) was dissolved in double-distilled water. TAU (alone or with BBBA) was mixed well with HPMC in hot water (70°) and homogenized thoroughly using a polytron homogenizer (Brinkmann Instruments, Westbury, NY). The final concentration of HPMC was 0.75%. The drug suspension was mixed well before and periodically during dosing. HPMC was preferred over the commonly used methylcellulose because the latter must be cooled to 10° in order to hydrate completely. Drugs were administered orally (0.1 mL/10 g) using 18 gauge intubation needles (Popper & Sons, Inc., New Hyde Park, NY). For i.p. injection, uridine was dissolved in normal saline solution (0.9% NaCl) and injected at 0.1 mL/10 g. To avoid a possible circadian variation in UrdPase activity [75, 76], drugs were administered between 8:30 and 9:00 a.m. Control mice received the carrier solution without the drug(s).

Collection of Samples

At various time intervals (5, 10, 15, 30 min, 1, 2, 3, 4, 6, 8, 12, and 24 hr) after drug administration, 250 μL of whole blood was collected from the orbital sinuses of each of five mice (lightly anesthetized with ethyl ether) in heparinized Natelson pipets and placed on ice [75]. The whole blood from each mouse was then centrifuged (Fisher Microcentrifuge model 235 A) at 12,400 rpm for 5 min, and the plasma was recovered and immediately stored in a −20° freezer until analysis by HPLC.

Preparation of the Samples

Plasma was allowed to thaw on ice and then was deproteinized with 2 vol. of 15% TCA. After centrifugation

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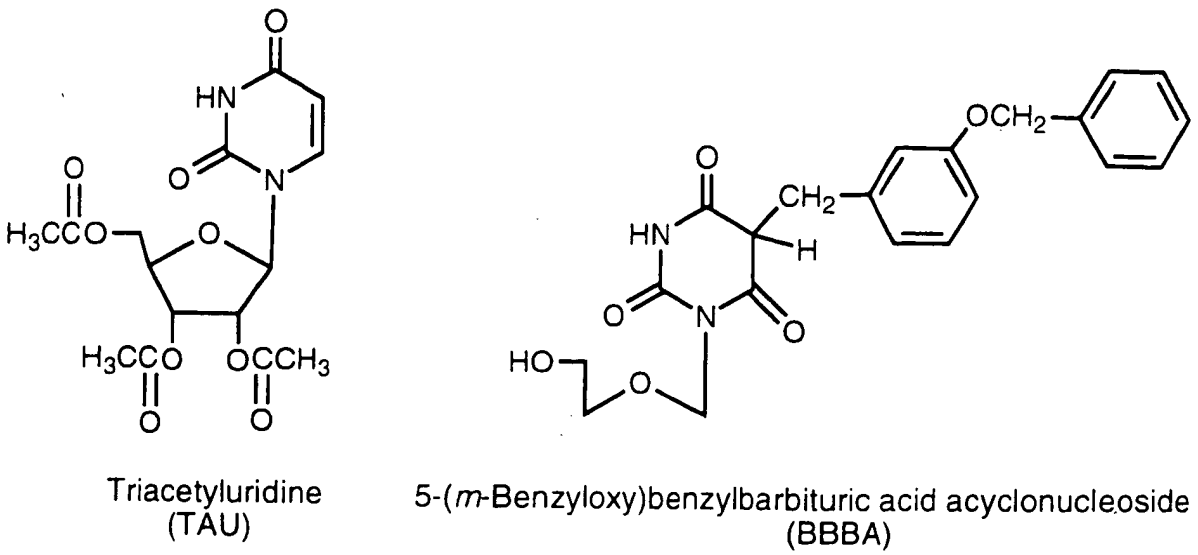

FIG. 1. Chemical structures of TAU and BBBA.

(16,000 *g*, 4°) for 5 min, using a Brinkmann Eppendorf Microcentrifuge, the supernatant acid-soluble material was neutralized by extraction with an equal volume of 1:2 mixture of tri-*n*-octylamine in freon. The neutralized supernatant was filtered through a Gelman Acrodisc LC 13 PVDF 0.2 μm filter, prior to HPLC analysis [75]. Under these conditions, the concentration of uridine released from TAU was not changed by the acid treatment or freezing during storage for up to 2 weeks (the longest duration of storage employed).

HPLC Analysis

Samples were analyzed by HPLC using a computer-controlled Hewlett-Packard model 1050 liquid chromatography apparatus equipped with an autosampler, a quaternary pump, and a multiple wavelength diode array base three channel UV detector. HPLC analysis was performed on two 5-μm Hypersil C_{18} reverse phase columns (250 × 5 mm) (Jones Chromatography, Littleton, CO) connected in tandem. Mobile phase was composed of two buffers, namely, Buffer A [50 mM ammonium acetate, 0.5% acetonitrile (pH 4.8)] and Buffer B [50 mM ammonium acetate, 60% acetonitrile (pH 4.8)]. Typically, 100 μL of treated plasma samples was analyzed with a multi-step elution protocol. A 23-min isocratic elution with Buffer A was followed by a 10-min linear gradient to 60% Buffer B, then a 22-min isocratic elution with 60% Buffer B, followed by a 20-min re-equilibration wash with 100% Buffer A. Flow rates were 1 mL/min, except for two 0.5 mL/min segments (8–23 min and 33–55 min). The effluent was monitored by UV absorption at 254 and 268 nm. Under these conditions, uracil, uridine, TAU and BBBA in the standards eluted at 13, 27, 47, and 48 min, respectively. Nevertheless, no TAU was recovered in plasma samples obtained as early as 5 min or at later time points. Instead, there were six metabolites, other than uridine and uracil, which we assumed to be the mono- and diacetyluridines. Incubation of TAU with plasma for various time periods supported this suggestion and showed the de-esterification of TAU to the six metabolites with measurable amounts of newly formed uridine but not uracil. BBBA, also, could not be recovered from plasma samples. A binding assay showed that BBBA has a high protein binding affinity. Only 0.5 and 6.5% of the drug were free after incubating 300 and 1000 μM concentrations, respectively, of BBBA with 25 mg/mL bovine serum albumin.

Uracil and uridine were identified by the ratio of their UV absorption at λ_{max} (259.5 and 262 nm, respectively)/254 nm, and co-elution with authentic samples. The recovery of uracil and uridine was more than 98% using [6-^{14}C]uracil and [2-^{14}C]uridine. The AUC values for uracil and uridine in the sample were calculated by the on-line computer. The concentrations of uracil or uridine in the samples were determined using standard curves for uracil or uridine prepared in double-distilled water. Plots of area under the curve versus uracil or uridine concentrations were linear between 1 and 5000 μM.

Pharmacokinetic Analysis of Plasma Uridine and Uracil

The pharmacokinetic parameters of uridine and uracil were estimated as previously described [48] by compartmental model-independent methods using a SIPHAR/BASE program [77]. The AUC was estimated by the trapezoidal rule with extrapolation to time infinity using the terminal disposition slope (K) generated by a weighed non-linear least-squares regression of an exponential fit of the data [78], with the weighed square factor set as the reciprocal of the calculated concentration squared. $T_{1/2}$ values of uridine were calculated from 0.693/K. The Cl_T was calculated by dividing the administered dose by the AUC and normalized to the weight of the animals. The apparent V_{ss} was calcu-

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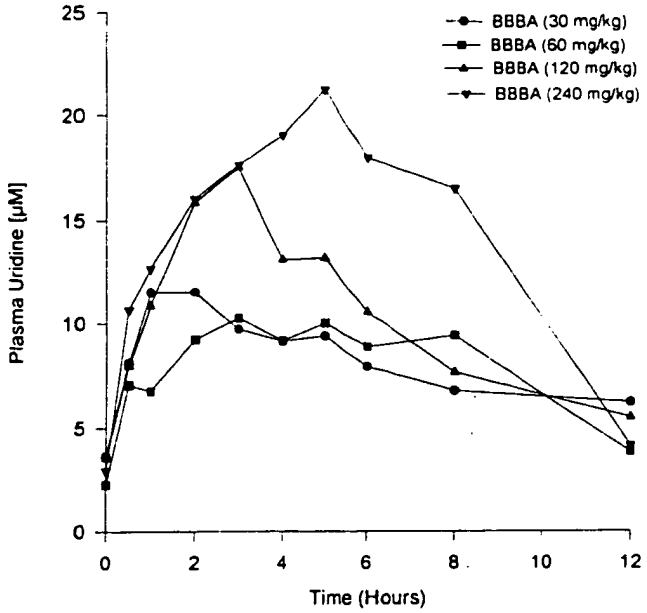

FIG. 2. Effect of different oral doses of BBBA on plasma uridine concentration in CD-1 mice. Each point represents the average from at least five mice.

lated as the product of the Cl_T and the MRT and normalized to the weight of the animals. The C_{max} and T_{max} values were estimated from the abscissa and the ordinate of the point with the highest ordinate on the computer-generated least squares curve depicting plasma concentration versus time. C_0 was the plasma concentration of endogenous uridine and uracil observed at zero-time (8:30 to 9:00 a.m.). Bioavailability of oral uridine was calculated as the percent of the AUC of plasma uridine resulting from oral administration of uridine/the AUC of plasma uridine resulting from i.p. administration of the same uridine dose. Relative bioavailability of uridine produced from the different oral regimens was expressed as the percent of the AUC of plasma uridine resulting from oral administration of uridine or TAU (alone or with BBBA)/the AUC of plasma uridine resulting from i.p. administration of the reference uridine concentration of 1320 mg/kg.

RESULTS

In the present study, the zero time (8:30 to 9:00 a.m.) concentrations of plasma uridine and uracil (C_0) in CD-1 mice were relatively constant, averaging 2.6 ± 0.7 and 7.4 ± 1.0 μM, respectively.

Administration of BBBA

Table 1 shows that oral administration of BBBA at 30, 60, 120, and 240 mg/kg increased the C_0 of plasma uridine by 3.2-, 4.6-, 5.4-, and 7.2-fold (C_{max}/C_0), respectively. Administration of the highest dose of BBBA (240 mg/kg) resulted in a plasma uridine C_{max} of 20.8 μM. Plasma uridine concentration remained 3- to 6-fold higher than control values for 8 hr after BBBA administration (Fig. 2). The AUC values were 104, 199, 227, and 280 μmol · hr/L, respectively (Table 1). There was no significant change in plasma uracil concentration following BBBA administration (data not shown).

Administration of Uridine

Previous studies have investigated the bioavailability and pharmacokinetics of wide range doses of oral uridine (350 to 5000 mg/kg) [48, 78]. Therefore, when we studied the effects of BBBA and TAU as modulators of plasma uridine concentration, we used only one dose of uridine as a reference dose for our investigations. We chose the uridine dose of 1320 mg/kg, which is approximately the median of previously studied doses. Administration of 1320 mg/kg of uridine by the i.p. route resulted in a uridine C_{max} of 2330 μM, a 1124-fold increase over zero time concentration (Table 2), at 5 min post-administration. However, this concentration dropped to 5 μM within 3 hr (data not shown). Plasma uracil concentration increased 61-fold, peaking to 536 μM at 0.6 hr (Table 2), and dropping to 19 μM within 3 hr, after which it was cleared from plasma (data not shown). The AUC values of plasma uridine and uracil were 1416 and 860 μmol · hr/L, respectively (Table 2).

Oral administration of the same dose of uridine (1320 mg/kg) resulted in a C_{max} of plasma uridine and uracil of 20 and 207 μM at 1.0 and 2.4 hr, respectively. Plasma uridine concentration remained at least 3-fold higher than basal concentration for up to 4 hr, while uracil was slowly cleared from plasma (Fig. 3A). The AUC values of plasma uridine and uracil were 109 and 1421 μmol · hr/L (Table 2). The $V_{d_{ss}}$ and the Cl_T of plasma uridine were 210 L/kg and 52 L/hr/kg, respectively. These values were 50- and 14-fold

TABLE 1. Effect of oral administration of different concentrations of BBBA on the pharmacokinetics of plasma uridine in CD1 mice

BBBA (mg/kg)	C_{max} (μM)	Fold change (C_{max}/C_0)	T_{max} (hr)	AUC (μmol · hr/L)
30	11.6 ± 3.7	3.18 ± 0.34	1.9 ± 0.14	104 ± 28.3
60	10.4 ± 3.6	4.61 ± 0.65	3.9 ± 1.28	199 ± 68.1
120	15.5 ± 3.4	5.39 ± 2.02	2.9 ± 0.66	227 ± 44.4
240	20.8 ± 11.2	7.19 ± 1.43	3.2 ± 0.04	280 ± 15.7

C_{max}, peak plasma concentration; C_0, zero time plasma concentration; T_{max}, time to peak plasma concentration; and AUC, area under the curve. Values are means ± SD from at least 5 mice at each time point.

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TABLE 2. Effect of administration of uridine alone and in combination with different concentrations of BBBA on the pharmacokinetics of plasma uridine and uracil in CD1 mice

Uridine (mg/kg)	BBBA	C_{max} (μM)	Fold change (C_{max}/C_0)	T_{max} (hr)	AUC (μmol·hr/L)	$V_{d_{ss}}$ (L/kg)	MRT (hr)	Cl_T (L/hr/kg)	$T_{1/2}$ (hr)
				Uridine					
Intraperitoneal									
1320	0	2330 ± 1120	1124 ± 112	0.08 ± 0.00	1416 ± 530	4.2 ± 3.1	1.1 ± 0.6	3.6 ± 1.3	0.29 ± 0.03
Oral									
1320	0	20.1 ± 4.2	7.5 ± 0.6	1.00 ± 0.28	109 ± 54.3	210.0 ± 13.9	4.6 ± 1.5	52.0 ± 8.5	0.26 ± 0.11
1320	30	74.2 ± 10.7	23.3 ± 1.8	1.00 ± 0.23	446 ± 164	55.7 ± 4.6	5.2 ± 1.2	12.4 ± 3.8	0.27 ± 0.05
1320	60	86.0 ± 35.7	28.2 ± 8.1	0.70 ± 0.23	577 ± 119	56.3 ± 22.2	6.2 ± 1.2	9.2 ± 1.5	0.14 ± 0.09
1320	120	141 ± 0.8	36.1 ± 9.9	0.41 ± 0.31	848 ± 259	36.1 ± 1.6	5.5 ± 1.5	6.3 ± 1.5	0.06 ± 0.05
				Uracil					
Intraperitoneal									
1320	0	536 ± 197.0	61.0 ± 26.3	0.55 ± 0.00	860 ± 362				
Oral									
1320	0	207 ± 80.0	27.1 ± 7.1	2.40 ± 0.09	1421 ± 519				
1320	30	179 ± 21.2	27.5 ± 1.4	1.75 ± 1.07	872 ± 880				
1320	60	40.6 ± 35.5	4.9 ± 3.5	3.00 ± 0.86	467 ± 57.4				
1320	120	29.1 ± 6.7	3.5 ± 0.4	1.75 ± 1.18	307 ± 16.3				

C_{max}, peak plasma concentration; C_0, zero time plasma concentration; T_{max}, time to peak plasma concentration; AUC, area under the curve; Cl_T, total plasma clearance; $T_{1/2}$, elimination half-life; $V_{d_{ss}}$, volume of distribution at steady state; and MRT, mean residence time. Values are means ± SD from at least 5 mice at each time point.

higher than those produced by the i.p. route (Table 2). The bioavailability of oral uridine was estimated to be 7.7%.

Administration of TAU

TAU was administered orally at 460, 1000, and 2000 mg/kg (molar equivalent to uridine doses of 300, 660, and 1320 mg/kg, respectively). The C_{max} of plasma uridine and uracil reached 78, 330, and 507 μM; and 265, 342, and 665 μM, respectively. These concentrations were 28-, 164-, and 252-fold, and 37-, 51-, and 72-fold higher than the C_0 of plasma uridine and uracil, respectively. The AUC values of plasma uridine were 82, 288, and 754 μmol·hr/L, while those of plasma uracil were 267, 610, and 2115 μmol·hr/L, respectively (Table 3 and Fig. 3B). The relative bioavailability of uridine released from oral TAU was 53%. Administration of TAU at the tested doses did not induce any noticeable toxicity (e.g. hypothermia, diarrhea, or weight loss) in the treated animals.

Coadministration of BBBA with Uridine

BBBA, at 30, 60, and 120 mg/kg increased the C_{max} of plasma uridine (20 μM), achieved by 1320 mg/kg of oral uridine alone, by 3.7-, 4.3-, and 7.0-fold, respectively (Table 2 and Fig. 3A). At the highest dose used (120 mg/kg), BBBA caused the plasma uridine C_{max} to reach 141 μM, 1 hr after coadministration, and to remain over 40 μM for 8 hr (Fig. 3A). Coadministration of 30, 60, and 120 mg/kg BBBA, increased also the AUC of plasma uridine (109 μmol·hr/L) by 4.1-, 5.3-, and 7.8-fold, respectively, and decreased the $V_{d_{ss}}$ (210 L/kg) as well as Cl_T (52 L/hr/kg) by 3.6-, 3.7-, and 5.8-fold, and 4.2-, 5.6- and 8.3-fold, respectively (Table 2). Thus, as shown in Fig. 4, coadministration of 30, 60, and 120 mg/kg BBBA increased the relative bioavailability of oral uridine from 7.7 to 31, 41, and 60%, respectively.

Plasma uracil concentration was also affected by the coadministration of BBBA: the higher the dose of BBBA, the lower the uracil concentration. Coadministration of BBBA at 30, 60, and 120 mg/kg with uridine decreased plasma uracil C_{max} from 207 to 179, 41, and 29 μM, respectively, with a corresponding reduction in the AUC from 1421 to 872, 467, and 307 μmol·hr/L, respectively (Table 2 and Fig. 3A).

Coadministration of BBBA with TAU

Coadministration of BBBA at 30, 60, and 120 mg/kg with TAU (460 mg/kg) increased the C_{max} of plasma uridine (78 μM), achieved by TAU alone, by 1.3-, 2.9-, and 2.5-fold, and reduced the C_{max} of plasma uracil (265 μM) by 5.2-, 8.7-, and 12.6-fold, respectively. BBBA also expanded the AUC of plasma uridine released from TAU (82 μmol·hr/L) by 1.7-, 4.4-, and 4.3-fold, respectively. This increase in plasma uridine AUC was accompanied by a concomitant 2.2-, 3.2-, and 3.6-fold decrease in plasma uracil AUC values (Table 3). A similar trend was observed when these same concentrations of BBBA were coadministered with a higher dose of TAU (1000 mg/kg). The C_{max} of plasma uridine (330 μM) was increased by 1.8-, 2.0-, and 3.2-fold, while that of uracil (342 μM) was decreased by 1.2-, 2.4-, and 3.0-fold, respectively. Consequently, the AUC of plasma uridine (288 μmol·hr/L) was increased by 2.4-, 2.8-, and 6.3-fold, while that of plasma uracil (610 μmol·hr/L) was reduced by a 1.1-, 1.3-, and 1.8-fold, re-

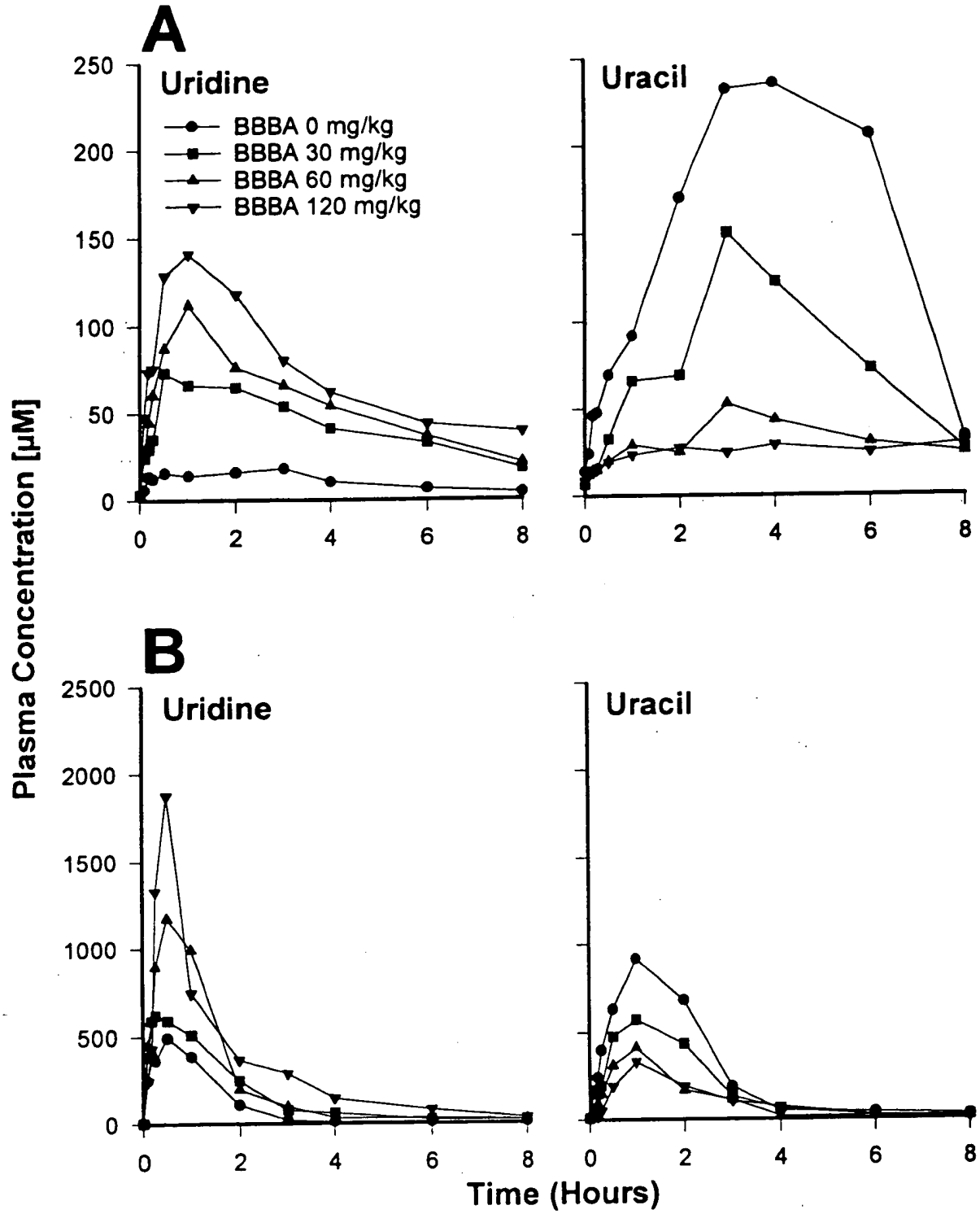

FIG. 3. Effect of oral coadministration of different doses of BBBA with (A) uridine (1320 mg/kg) or (B) a molar equivalent dose of TAU (2000 mg/kg) on plasma concentration of uridine and uracil in CD-1 mice. Each point represents the average from at least five mice.

spectively. At the highest dose of TAU (2000 mg/kg) tested, coadministration of BBBA further increased he C_{max} of plasma uridine (507 μM) by 1.2-, 2.3-, and 3.2-fold and decreased that of uracil (665 μM) by 1.2-, 2.1-, and 2.5-fold, respectively. Coadministration of BBBA also increased the AUC of plasma uridine (754 μmol · hr/L) by 1.6-, 2.7-, and 3.9-fold and decreased that of uracil (2115 μmol · hr/L) by 1.4-, 2.4-, and 2.7-fold, respectively. Figure 4 shows the effects of different doses of BBBA on the relative bioavailability of uridine released from oral TAU.

DISCUSSION

The present results indicated that the bioavailability of oral uridine is only 7.7%. This is in agreement with other results

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TABLE 3. Effect of oral administration of TAU alone and in combination with BBBA on the pharmacokinetics of plasma uridine and uracil in CD1 mice

TAU (mg/kg)	BBBA (mg/kg)	C_{max} (μM)	Fold change (C_{max}/C_0)	T_{max} (hr)	AUC (μmol· hr/L)
		Uridine			
460	0	78.2 ± 21.8	28.2 ± 1.8	0.20 ± 0.04	82.4 ± 41.0
460	30	104 ± 30.9	36.6 ± 9.9	0.13 ± 0.02	144 ± 72.9
460	60	225 ± 68.3	105 ± 14.3	0.25 ± 0.03	366 ± 32.6
460	120	192 ± 13.4	95.6 ± 29.9	0.45 ± 0.14	358 ± 10.4
1000	0	330 ± 102	164 ± 10.5	0.25 ± 0.04	288 ± 85.4
1000	30	582 ± 256	290 ± 55.1	0.25 ± 0.11	693 ± 118
1000	60	670 ± 95	333 ± 104	0.25 ± 0.03	796 ± 73.2
1000	120	1045 ± 329	520 ± 325	0.50 ± 0.03	1814 ± 994
2000	0	507 ± 298	252 ± 22.3	0.41 ± 0.22	754 ± 355
2000	30	621 ± 223	309 ± 128	0.31 ± 0.04	1239 ± 468
2000	60	1173 ± 503	584 ± 19.5	0.50 ± 0.02	2016 ± 493
2000	120	1618 ± 706	805 ± 17.8	0.50 ± 0.09	2931 ± 824
		Uracil			
460	0	265 ± 70.4	37.1 ± 21.5	0.29 ± 0.02	267 ± 50.8
460	30	50.8 ± 42.2	8.3 ± 5.0	0.46 ± 0.09	123 ± 77.0
460	60	30.4 ± 6.1	6.1 ± 0.3	0.45 ± 0.02	84.3 ± 20.8
460	120	21.0 ± 3.7	3.5 ± 0.1	0.25 ± 0.10	74.4 ± 18.5
1000	0	342 ± 79.9	50.7 ± 2.0	0.62 ± 0.00	610 ± 158
1000	30	275 ± 51.4	40.7 ± 0.3	0.52 ± 0.03	536 ± 98.1
1000	60	141 ± 24.6	28.2 ± 9.9	0.70 ± 0.01	466 ± 91.0
1000	120	112 ± 94.4	24.2 ± 14.8	0.69 ± 0.10	338 ± 125
2000	0	665 ± 287	71.6 ± 13.9	1.00 ± 0.10	2,115 ± 839
2000	30	536 ± 190	57.7 ± 8.6	0.76 ± 0.17	1,466 ± 375
2000	60	313 ± 130	44.2 ± 5.7	0.76 ± 0.03	892 ± 405
2000	120	266 ± 56.8	36.3 ± 5.3	1.00 ± 0.03	775 ± 208

C_{max}, peak plasma concentration; C_0, zero time plasma concentration; T_{max}, time to peak plasma concentration; and AUC, area under the curve. Values are means ± SD from at least 5 mice at each time point.

from mice [79] and humans [45]. The present data also demonstrated that TAU is a superior substitute for uridine. The relative bioavailability of uridine following oral TAU administration (53%) was 7-fold higher than that achieved by oral uridine (7.7%). Not only did TAU increase the concentration of plasma uridine, but it also reduced the time required to attain the maximum concentration. Oral TAU produced a plasma uridine C_{max} of 507 μM at 0.4 hr, while an equimolar dose of oral uridine resulted in a C_{max} of only 20 μM at 1 hr (Tables 2 and 3).

The low bioavailability of oral uridine can be attributed mainly to the first pass effect and reflects the contribution of the intestine and liver to uridine catabolism. It was reported previously that there is an inverse relationship between plasma uridine concentration and its hepatic clearance, i.e. increasing the uridine concentration entering the liver is accompanied by a decrease in hepatic clearance [63], until uridine reaches a concentration of approximately 50 μM (the threshold or hepatic maximum for uridine clearance), after which a constant amount of uracil is discharged into the circulation [63]. This threshold or hepatic maximum for uridine clearance results from saturation of the transport system in the liver and/or catabolism of uridine by hepatic UrdPase activity [63]. These factors could also apply to the intestine which is a major organ responsible for the low bioavailability of oral uridine [45, 79, and the present results]. In this regard, it should be noted that UrdPase activity in the intestine is the highest in all studied organs of the body. In mice, intestinal UrdPase activity (47,308 ± 1,498 pmol/min/mg protein) was 146-fold higher than that of the liver (unpublished results). Such high activity of uridine catabolism in the intestine and liver is considered among the principal components of the rapid disappearance of uridine from plasma following its oral administration [48, 55, 58, 62]. This view is supported by the respective 50- and 15-fold increase in the V_{dss} and Cl_T of oral uridine when compared to i.p. uridine (Table 2).

The better efficiency of oral TAU over uridine in delivering uridine to the plasma can also be ascribed to the extent of the extravascular catabolism of uridine by UrdPase. TAU, unlike uridine, is resistant to catabolism by UrdPase. It is also more lipophilic which enhances its absorption from the gastrointestinal tract and reabsorption from the renal tubules [70]. Therefore, a large portion of administered TAU is transported or diffused into the plasma unchanged and/or as mono- or diester derivatives. In plasma, these uridine esters act as depots releasing uridine by plasma esterases over a longer period of time than when oral uridine is used. Furthermore, our unpublished results indicate that, in contrast to other sites in the body, plasma has a negligible UrdPase activity (6 pmol/min/mg protein). Indeed, incubation of TAU with plasma resulted in the lib-

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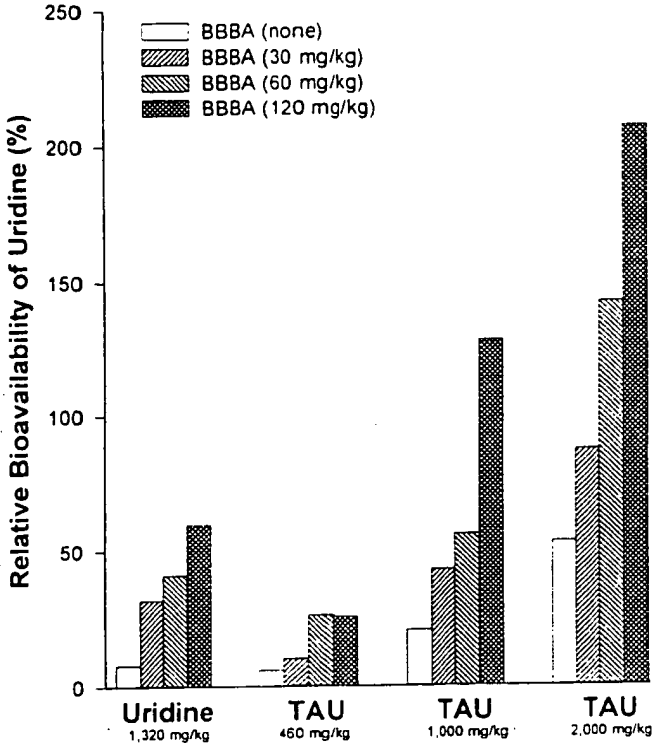

FIG. 4. Relative bioavailability of uridine from the oral administration of uridine or TAU, alone or with different doses of BBBA in CD-1 mice. Relative bioavailability is calculated as the percent of AUC of plasma uridine resulting from the oral administration of the compound(s) under study/AUC of plasma uridine resulting from the i.p. administration of 1320 mg/kg uridine. Each value represents the average from at least five mice.

eration of the mono- and diacetyl derivatives of TAU as well as uridine, but not uracil. Hence, higher and sustained levels of plasma uridine were observed after administration of TAU than when uridine was used. These results suggest that the difference between oral uridine and TAU in delivering uridine to the plasma is due primarily to extravascular uridine catabolism by UrdPase. This postulation is further supported by the finding that coadministration of the UrdPase inhibitor BBBA (120 mg/kg) with uridine enhanced the relative bioavailability of oral uridine by 7.8-fold, while its coadministration with an equimolar concentration of TAU increased the relative bioavailability of plasma uridine by only 3.9-fold (Fig. 4).

BBBA, the most potent inhibitor of UrdPase [71–73], is a powerful enhancer of plasma uridine concentration. Oral administration of BBBA produced a dose-dependent increase in the AUC and C_{max} of plasma uridine (Table 1). Although uridine C_{max} achieved by BBBA was similar to that attained by oral administration of another UrdPase inhibitor, 5-benzylacyclouridine (BAU) [49, 80], the effect of BBBA on plasma uridine concentration was more prolonged. BBBA at 120 and 240 mg/kg maintained plasma uridine concentration 3- and 6-fold higher than the zero time concentration for 8 hr after administration (Fig. 2). On the other hand, uridine concentration dropped to near zero time concentration 6 hr post-administration of the same doses of BAU [49, 80].

Coadministration of BBBA with uridine or TAU increased the relative bioavailability of uridine in a dose-dependent fashion (Fig. 4), presumably due to inhibition of UrdPase as indicated by the increase in the AUC, C_{max} and C_{max}/C_0 and decrease in $V_{d_{ss}}$ and Cl_T of plasma uridine, as well as decrease in the AUC and C_{max} of plasma uracil (Tables 2 and 3 and Fig. 3). However, the combination of BBBA with TAU was superior to that with uridine in increasing plasma uridine concentration and bioavailability. Figure 4 demonstrates that coadministration of BBBA (30, 60, and 120 mg/kg) with uridine (1320 mg/kg) increased the relative bioavailability of oral uridine (7.7%) by 4.1-, 5.3-, and 7.8-fold, while coadministration of the same doses of BBBA with a molar equivalent dose of oral TAU (2000 mg/kg) improved the relative bioavailability of uridine released from TAU (53%) by 1.7-, 2.7-, and 3.9-fold, respectively. The superiority of the BBBA and TAU combination is also evident from the fact that the AUC of plasma uridine observed after administration of 1320 mg/kg uridine with 120 mg/kg BBBA could be achieved by the administration of TAU (1000 mg/kg) with BBBA (60 mg/kg), i.e. the molar equivalent of half the doses of uridine and BBBA, respectively.

The marked increase of plasma uracil concentration following the administration of uridine or TAU (Tables 2 and 3) could be attributed to the saturation of uracil catabolism. DHUDase (EC 1.3.1.2), the rate-limiting enzyme of uracil catabolism in the liver [81], is a saturable enzyme and inhibited by increasing concentration of its substrate, uracil [81]. Degradation by UrdPase of a large amount of the administered uridine or the uridine released from TAU would increase uracil formation. When uracil concentration reaches the critical saturating limit (*ca.* 75 μM), it inhibits DHUDase [81]. This would lead to the delivery of increasing amounts of uracil to the plasma, hence, the observed rise in plasma uracil concentration and AUC after administration of uridine or TAU. It should be noted, however, that oral TAU increased the C_{max} and AUC of plasma uracil above that achieved by administration of an equimolar dose of oral uridine. This observation is not unexpected since administered uridine is subject to the sequential activities of intestinal and hepatic UrdPase and DHUDase. These activities would lead not only to reduction of uridine bioavailability but also to a decreased uracil pool. On the other hand, TAU and its mono- and diacetyl derivatives are not subject to UrdPase activity, hence the expansion of available uridine which will be reabsorbed and eventually metabolized to increase plasma uracil concentration.

The lack of significant alterations in plasma uracil concentration following the administration of BBBA alone indicates that the doses of BBBA used were not sufficient to inhibit totally UrdPase and uridine catabolism. Consequently, the remaining UrdPase activity converts uridine to

uracil, which in turn is subject to DHUDase activity. However, the concentration of this newly formed uracil appears not to be high enough to disturb the homeostatic mechanisms maintaining the constancy of plasma uracil concentration, including the saturation and inhibition of DHUDase. As a result, plasma uracil concentration remained unchanged.

In conclusion, the specific UrdPase inhibitor BBBA alone increased plasma uridine concentration and bioavailability in a dose-dependent manner. TAU, a prodrug of uridine, proved to be an excellent substitute for uridine in achieving a greater bioavailability of plasma uridine. Combining BBBA with uridine or TAU, for oral administration, secured and maintained higher levels of plasma uridine than either alone. However, the combination of BBBA plus TAU was more effective in accomplishing this goal. Therefore, the combination of BBBA plus TAU can provide a better substitute for the massive doses of uridine required to achieve the high levels of uridine necessary to rescue or protect from host-toxicities of certain anti-cancer and anti-viral pyrimidine analogues, without the toxic side-effects associated with such doses of uridine. The combination of BBBA plus TAU can also replace uridine in the treatment of other pathological disorders that can be remedied by administration of uridine.

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Phase I and Pharmacologic Study of PN401 and Fluorouracil in Patients With Advanced Solid Malignancies

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Purpose: To assess the feasibility of administering PN401, an oral uridine prodrug, as a rescue agent for the toxic effects of fluorouracil (5-FU), and to determine the maximum-tolerated dose of 5-FU when given with PN401, with an 8-hour treatment interval between these agents.

Patients and Methods: Patients with advanced solid malignancies were treated with escalating doses of 5-FU, given as a rapid intravenous infusion weekly for 3 consecutive weeks every 4 weeks. PN401 was administered orally 8 hours after 5-FU administration, to achieve sustained plasma uridine concentrations of at least 50 μmol/L. Initially, patients received 6 g of PN401 orally every 8 hours for eight doses (schedule 1). When dose-limiting toxicity (DLT) was consistently noted, patients then received 6 g of PN401 every 2 hours for three doses and every 6 hours thereafter for 15 doses (schedule 2).

Results: Twenty-three patients received 50 courses of 5-FU and PN401. Among patients on schedule 1, DLT (grade 4 neutropenia complicated by fever and diarrhea) occurred in those receiving 5-FU 1,250 mg/m²/wk. Among patients on schedule 2, 5-FU 1,250 mg/m²/wk was well tolerated, but grade 4, protracted (> 5 days) neutropenia was consistently noted in those treated with higher doses of the drugs. Nonhematologic effects were uncommon and rarely severe. The pharmacokinetics of 5-FU, assessed in 12 patients on schedule 2, were nonlinear, with the mean area under the time-versus-concentration curve (AUC) increasing from 298 ± 44 to 962 ± 23 μmol/L and mean clearance decreasing from 34 ± 4 to 15.6 ± 0.38 L/h/m² as the dose of 5-FU was increased from 1,250 to 1,950 mg/m²/wk. 5-FU AUCs achieved with 5-FU 1,250 mg/m²/wk for 6 weeks along with the intensified PN401 dose schedule were approximately five-fold higher than those achieved with 5-FU alone. Plasma uridine concentrations increased with each of the three PN401 doses given every 2 hours, and uridine steady-state concentrations were greater than 50 μmol/L.

Conclusion: Treatment with oral PN401 beginning 8 hours after 5-FU administration is well tolerated and results in sustained plasma uridine concentrations above therapeutic-relevant levels. The recommended 5-FU dosage for phase II evaluations is 1,250 mg/m²/wk for 3 weeks every 4 weeks with the intensified PN401 dose schedule (schedule 2). At this dose, systemic exposure to 5-FU as measured by AUC was five-fold higher than that observed after administration of a conventional 5-FU bolus.

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FLUOROURACIL (5-FU) IS one of the most widely used antineoplastic agents and the mainstay of chemotherapy for gastrointestinal and other types of cancers.[1] The principal mechanisms of 5-FU cytotoxicity include inhibition of thymidylate synthase, largely through the actions of its metabolite, fluorodeoxyuridine monophosphate (FdUMP); and inhibition of RNA synthesis as a result of incorporation of a second metabolite, fluorouridine triphosphate (FUTP), into RNA.[2] The principal toxicities of 5-FU are leukopenia, mucositis, diarrhea, and hand-foot syndrome, with the latter two adverse effects predominating when 5-FU is administered as a continuous intravenous (IV) infusion.[3] Like other conventional cytotoxic antineoplastic agents, 5-FU has a relatively narrow therapeutic index, in that toxicity often limits the dose of 5-FU that can be administered, as well as its overall therapeutic usefulness.

Uridine, a naturally occurring pyrimidine nucleoside, selectively reduces incorporation of FUTP into the RNA of hematopoietic progenitor and gastrointestinal mucosal cells, thereby preventing 5-FU toxicity in normal tissues.[4-8] In mice, administration of uridine after treatment with 5-FU reduces toxicity to normal tissues, permits substantial 5-FU dose escalation, and increases overall efficacy of 5-FU.[4-8] Results of preclinical and clinical studies indicate that uridine concentrations of at least 50 μmol/L are sufficient to confer protection to normal tissues from the toxic effects of 5-FU.[6] Differences in uptake and use of uridine between

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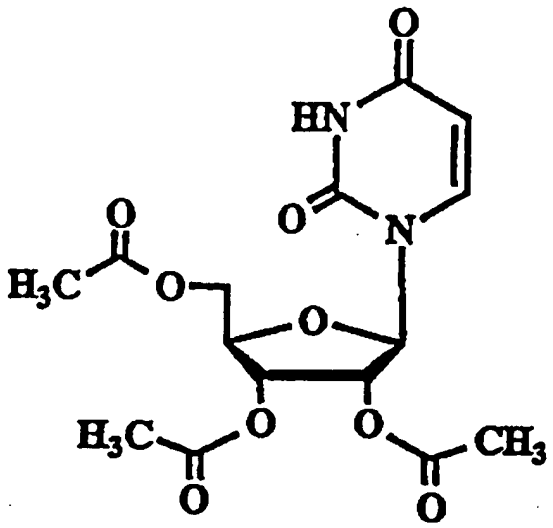

Fig 1. Structure of PN401.

tumor and normal tissues lie behind uridine's ability to reduce the toxicities of 5-FU without proportionally reducing antitumor activity.[3] Both hematopoietic progenitors and gastrointestinal mucosa stem cells efficiently incorporate exogenous uridine (salvage pathway), whereas most other tissues, including malignant tumors, favor the de novo pathway of uridine nucleotide biosynthesis, in which free uridine is not an intermediate.[3] Although uridine has also been demonstrated to protect against 5-FU toxicity in humans, its low and erratic oral bioavailability and the requirement for central venous access for parenteral administration preclude clinical utility.[9-12]

PN401 (2',3',5'-tri-*O*-acetyluridine; Pro-Neuron, Inc, Gaithersburg, MD) (Fig 1) is an orally active prodrug of uridine that is efficiently absorbed from the gastrointestinal tract and deacetylated by nonspecific esterases, yielding uridine and free acetate. In contrast to oral uridine, PN401 is not a substrate for the catabolic enzyme uridine phosphorylase and does not require the pyrimidine transporter for absorption. Consequently, administration of PN401 results in substantially more bioavailable uridine than does oral administration of uridine itself. In an earlier phase I study of 5-FU given weekly in combination with PN401, in which PN401 was administered every 6 hours for 10 doses beginning 24 hours after 5-FU administration, PN401 doses as high as 9.9 g were well tolerated.[13] Treatment with PN401 alone increased plasma uridine concentrations from pretreatment levels ranging from 3 to 6 μmol/L to peak concentrations averaging 167.6 ± 36.9 μmol/L. After multiple doses of PN401, uridine trough concentrations averaged 67.1 ± 19.1 μmol/L. Plasma uridine concentrations readily exceeded 50 μmol/L (the concentration that has consistently been demonstrated to protect normal tissues from the toxic effects of 5-FU) for more than 6 hours.[13] In that study, PN401 at its recommended dose of 6 g substantially reduced the incidence and severity of 5-FU toxicity, permitting an increase of the 5-FU dose to 1,000 mg/m^2/wk for 6 consecutive weeks.[13]

Preclinical studies have indicated that timing of the first dose of PN401 relative to 5-FU administration is an important determinant of the effectiveness of PN401 in ameliorating the adverse effects of 5-FU. For example, treatment of mice bearing colon 26 tumors with PN401 2 hours after 5-FU administration resulted in substantially greater antitumor efficacy and less toxicity compared with treatment of mice with PN401 24 hours after 5-FU administration.[4] These results suggest that the clinical utility of PN401 can be optimized by administering the agent soon after treatment with 5-FU. Therefore, in this phase I pharmacokinetic study, we evaluated the feasibility of administering high doses of 5-FU on a weekly schedule in combination with PN401, administered starting 8 hours after treatment with 5-FU, unlike in previous investigations in which PN401 was given 24 hours after 5-FU administration.[13] Additionally, because of findings that plasma uridine concentrations exceed biologically relevant levels of 50 μmol/L for more than 6 hours, PN401 was initially administered every 8 hours instead of at 6-hour intervals.[13]

The principal objectives of the present study were to determine the maximum-tolerated dose (MTD) of 5-FU administered as a 30-minute IV infusion weekly for 3 weeks every 4 weeks with PN401 and to recommend doses of these agents for subsequent phase II trials; to characterize the principal toxicities of the regimen; to describe the pharmacokinetic behavior of 5-FU administered with PN401 in this schedule; and to obtain preliminary evidence of antitumor activity, if any.

PATIENTS AND METHODS

Eligibility

Patients with histologically documented advanced solid malignancies refractory to conventional therapy or for whom no effective therapy existed were candidates for this study. Eligibility criteria included age ≥ 18 years; Eastern Cooperative Oncology Group performance status ≤ 2; life expectancy greater than 12 weeks; no prior chemotherapy or radiation therapy within 4 weeks of entering onto the study (6 weeks for nitrosoureas and mitomycin); adequate hematopoietic function (WBC count ≥ 3,500/μL, absolute neutrophil count [ANC] ≥ 1,500/μL, and platelet count ≥ 100,000/μL), hepatic function (total bilirubin level ≤ 1.5 mg/dL and AST and ALT levels < two times the upper limit of normal [< five times the upper limit of normal for patients with liver metastasis]), and renal function (creatinine level ≤ 1.5 mg/dL); no active infection or other coexisting

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medical problems severe enough to limit compliance; no malabsorption syndrome or other condition that might interfere with intestinal absorption; and documentation of tolerance to treatment with 5-FU or peripheral-blood mononuclear-cell dihydropyrimidine dehydrogenase (DPD) activity of at least 0.12 nmol/min/mg protein. Before treatment, all patients gave written informed consent according to federal and institutional guidelines.

Dosage and Drug Administration

PN401 was supplied by Pro-Neuron, as white tablets containing 0.5 g of PN401. 5-FU (Pharmacia & Upjohn, Kalamazoo, MI) came in ampules containing 50 mg/mL.

5-FU, administered IV over 30 minutes, was given weekly for 3 weeks every 4 weeks. PN401 was administered at a fixed oral dose starting 8 hours after 5-FU administration. Initially, patients received 6 g of PN401 (12 tablets) every 8 hours for eight doses (schedule 1). After dose-limiting toxicity (DLT) was noted on this schedule, the PN401 dose schedule was modified to 6 g every 2 hours for three doses followed by 6 g every 6 hours for 15 doses (schedule 2). Patients were instructed to take PN401 with water and to repeat the dose if they vomited within 2 hours after ingestion. Patients were asked to record the precise times and numbers of PN401 pills taken in diaries that were collected and reviewed after each course.

The starting dose of 5-FU was 1,000 mg/m^2. This dose was increased by 25% increments in groups of new patients if the previous dose level was well tolerated. One new patient was treated at each successively higher dose level unless toxicity of at least grade 2 severity was experienced during the first course of treatment. In the event of toxicity of at least grade 2 severity, a minimum of two additional new patients were entered at that dose level. If none of the three patients experienced DLT during course 1, dose escalation resumed, with a minimum of three new patients treated at each successive dose level. If DLT was observed during the first course of treatment in any patient at any dose level, as many as six new patients were treated at that dose level. The MTD and recommended phase II dose were defined as the highest 5-FU dose at which fewer than two of six new patients experienced DLT during course 1. DLT was defined as grade 3 nonhematologic toxicity (excluding nausea or vomiting), any grade 4 nonhematologic toxicity, grade 4 thrombocytopenia (platelet count < 25,000/μL), severe anemia (hemoglobin level < 6.5 g/dL), and grade 4 neutropenia (ANC < 500/μL) lasting more than 5 days and/or associated with fever. Toxicities were graded using the National Cancer Institute common toxicity criteria.[14]

The weekly dose of 5-FU was reduced by one dose level in patients who experienced either grade 2 nonhematologic or grade 2 or 3 hematologic toxicity on the day of scheduled treatment. 5-FU was not administered to patients who experienced grade 4 hematologic toxicity or grade 3 or 4 nonhematologic toxicity on the day of planned treatment, and the omitted dose was not made up. When treatment was resumed, the dose of 5-FU was reduced by one level for the remainder of treatment. Treatment delays that exceeded 2 weeks because of failure to return to a grade 0 or 1 toxicity level mandated a 5-FU dose reduction by one level for the remainder of treatment unless the toxicity recurred, in which case a second dose reduction was required. The dose of PN401 was not modified in cases of toxicity.

Pretreatment and Follow-Up Studies

Histories, physical examinations, and routine laboratory evaluations were performed before treatment and weekly. Routine laboratory evaluations included complete blood counts; differential WBC counts; determination of electrolyte levels; measurement of blood urea nitrogen, creatinine, glucose, total protein, albumin, calcium, phosphate, uric acid, alkaline phosphatase, total and direct bilirubin, AST, and ALT levels; determination of prothrombin time; and urinalysis. DPD activity in the peripheral mononuclear cells of patients who had never been treated with 5-FU was measured in the laboratory of Robert Diasio, MD, at the University of Alabama at Birmingham as previously described.[15] Pretreatment studies also included a chest x-ray and relevant radiologic studies for evaluation of all measurable or assessable sites of malignancy. These studies were repeated after every other course. Patients were able to continue treatment if they did not develop progressive disease. A patient was said to have a complete response if two studies at least 4 weeks apart showed disappearance of all active disease, and a patient with a partial response had at least a 50% reduction in the sum of the product of the bidimensional measurements of all lesions documented, with sets of measurements being performed at least 4 weeks apart. Any concurrent increase in the size of any lesion by 25% or more or the appearance of any new lesion was considered disease progression.

Plasma Sampling and Assay

To study the pharmacokinetic behavior of 5-FU and PN401, we obtained blood from a site contralateral to the peripheral vein used for treatment. Blood samples were collected before treatment with 5-FU, immediately after the 30-minute infusion, and 10, 45, 90, and 180 minutes after treatment on day 1 of course 1. To determine the concentration of uridine in plasma after treatment with PN401 on the more intensive PN401 dose schedule (schedule 2), we obtained blood before the second and third doses, 2 hours after the third dose, and immediately before the fourth dose.

The blood samples collected for 5-FU analysis were centrifuged immediately after collection and stored at −20°C until analysis. Plasma (250 μL), thawed from −20°C, was transferred to a 16 × 125-mm silicon tube, and 30 μL of 5-bromouracil, which served as an internal standard, was added. To precipitate proteins, we added 500 mL of 10 mmol/L ammonium sulfate solution, followed by 4 mL of ethyl acetate and isopropanol (90/10 [vol/vol]). The mixture was vortexed for 45 minutes, and the tube was centrifuged for 5 minutes. The organic layer was pipetted into a 13 × 100-mm disposable Pyrex culture tube and dried in a 50°C water bath. The extraction procedure was repeated twice, and each tube was reconstituted with 200 μL of 0.01 mol/L potassium phosphate at pH 4, which was the mobile phase for high-performance liquid chromatography (HPLC).

HPLC was performed using a Spectra-Physics Isochrome pump (Spectra-Physics, Mountain View, CA) connected to a Hitachi AS4000 autoinjector (Hitachi, San Jose, CA) and an SP8490 variable-wavelength fluorescence detector (Spectra-Physics) set at 260 nm. After injection of a 20-μL sample, 5-FU and 5-bromouracil were separated using a YMC-Pack, ODS-AMQ (4.6 × 250-mm, 5-μm) C_{18} column (Yamamura Chemical Co, Kyoto, Japan) using a mobile phase of 0.01 mol/L potassium phosphate, pH 4, and a flow rate of 2.0 mL/min. The retention times of 5-FU and 5-bromouracil were 3.43 and 7.5 minutes, respectively. The standard curve for 5-FU was prepared over a range of 0.246 to 999.4 μmol/L by adding known amounts of 5-FU and internal standard to appropriate volumes of human plasma. A calibration curve was generated by linear regression of the peak height ratio of the 5-FU concentration to that of the internal standard. The values for the intraday and interday precision for this method were less than 2%, and the lower limit of quantification of 5-FU was 0.2 μmol/L based on the extraction of 250 mL of plasma.

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Plasma uridine concentrations were determined using reverse-phase HPLC that was validated using inosine as an internal standard. Blood samples were centrifuged immediately after collection and stored at −20°C until analysis. Plasma (500 μL), thawed from −20°C, was transferred to a 1.5-mL siliconized centrifuge tube containing 300 mL of water. An aliquot of 20 μL of the internal standard (600 μg/mL inosine in 10% methanol) was added to all control samples, followed by 200 μL of 40% trichloroacetic acid. The tubes were then vortexed completely using a vortex mixer. The sealed tube was next placed in an ice-water bath for 10 to 30 minutes to precipitate plasma proteins. The trichloroacetic acid was extracted with 4 mL of methyl-*tert*-butyl ether, and the top ether layer was removed. Two hundred microliters of the underlayer was placed in an autosampler microvial. Analysis of the extracted samples was performed on a HPLC system that consisted of a Spectra-Physics Isochrome pump (Spectra-Physics) connected to a Hitachi AS4000 autoinjector (Hitachi) and an SP8490 variable-wavelength fluorescence detector (Spectra-Physics) set at 260 nm. After injection of 10 to 50 μL of sample, uridine was separated by a Kromasil (4.6 × 250-mm, 5-μm) C_{18} column (Eka Nobel, Bohus, Sweden) using a mobile phase consisting of a mixture of 20 mmol/L sodium acetate at pH 4.5 and 5% methanol at a flow rate of 1.0 mL/min. The retention times of uridine and inosine were 6 and 11 minutes, respectively. The standard curve for uridine was prepared over a range of 0.5 to 1,200 μmol/L by adding known amounts of uridine and internal standard to appropriate volumes of human plasma. A calibration curve was generated by linear regression of the peak height ratio of uridine to the internal standard versus uridine concentration. The values of intraday and interday precision for this method were less than 2%, and the lower limit of quantification of uridine was 1.31 μmol/L based on the extraction of 500 μL of plasma.

Pharmacokinetic and Pharmacodynamic Analysis

Plasma 5-FU concentration data were analyzed by standard noncompartmental pharmacokinetic methods using the program WinNonlin (Statistical Consultants, Inc, Apex, NC). The area under the concentration-versus-time curve (AUC) was calculated using the linear trapezoidal rule. The AUC was extrapolated to infinity by dividing the last measured concentration by the elimination-rate constant, k_e, which was estimated by log-linear fit of the terminal portion of the curve. The portion of the total AUC ($AUC_{0-\infty}$) calculated by extrapolation was less than 5%. The systemic clearance (Cl) was determined by dividing the dose by the AUC, the elimination half-life was calculated by dividing 0.693 by the k_e, and the apparent volume of distribution (V_d) was calculated using the formula $V_d = dose/k_e \times AUC$. The maximum plasma concentration (C_{max}) was determined by inspection of the concentration-versus-time curve. Pharmacokinetic parameters were described using descriptive statistics. Standard linear regression methods were used to evaluate the relationships between dose and pharmacokinetic parameters to determine whether 5-FU pharmacokinetics were dose independent.

The peak plasma uridine concentrations after PN401 administration were determined by visual inspection of the uridine concentration–versus-time curves. The mean plasma uridine concentration was calculated as the arithmetic mean of the plasma uridine concentration values obtained for each patient after administration of the first three doses of PN401. Plasma uridine concentrations after administration of PN401 were compared with predicted values obtained by applying the principle of superposition to the plasma concentration–versus-time curve of uridine observed in healthy subjects after a single 6-g oral dose of PN401.[15] With this method, the AUC, Cl, V_d, half-life, and k_e of uridine after treatment with a single 6-g oral dose of PN401 in healthy volunteers are estimated by a one-compartment open model with first-order absorption kinetics using the program WinNonlin (Statistical Consultants). The estimated parameters are subsequently used to simulate the plasma concentration–versus-time curve of doses of 6 g of PN401 every 2 hours. It is assumed that the pharmacokinetic behavior of uridine after each dose of PN401 is not affected by other doses and that the absorption, conversion of PN401 to uridine, and systemic clearance of uridine do not change with repetitive dosing.

We explored the relationships between toxicity and pharmacokinetic parameters reflecting systemic exposure to both 5-FU and uridine. The percentage decrements in ANCs and platelet counts, as well as the occurrence of DLT, were related to the dose, $AUC_{0-\infty}$ and C_{max} of 5-FU and to both the peak and mean plasma uridine concentrations. The percentage decrement in blood cell counts was calculated as follows:

$$\textit{Percentage decrement in blood count} = [100\% \times (\textit{pretreatment count} - \textit{nadir count})]/\textit{pretreatment count}$$

The nonparametric Mann-Whitney *U* test and Kruskal-Wallis test were used to compare pharmacokinetic parameters reflecting drug exposure in patients with different grades of toxicity. Linear and nonlinear regression methods were used to assess the relationships between quantitative parameters of myelosuppression and relevant parameters of drug exposure.

RESULTS

Twenty-three patients, whose characteristics are listed in Table 1, were treated with a total of 50 courses of 5-FU and PN401 through four dose levels (Table 2). One patient discontinued therapy in the middle of the first course of 5-FU 1,250 mg/m^2 and PN401 and was considered unassessable. Twenty-one patients had received previous chemotherapy and 14 patients had previously been treated with 5-FU–containing regimens. The median number of courses administered per patient was two (range, one to six). Four patients required either a reduction (one patient) or omission (three patients) of the weekly dose of 5-FU, and doses were reduced in six patients because of intolerable toxicity in the prior course. Seven patients required 1-week delays in treatment, and a single patient required a 2-week delay because of incomplete recovery of blood cell counts. Major objective antitumor responses were not observed.

PN401 was well tolerated on both dose schedules, and no adverse effects were directly attributed to this agent. On the basis of reviews of patient diaries and interval histories, there was no evidence of noncompliance with PN401 treatment. The rate of DLTs, particularly adverse gastrointestinal and hematologic toxicities, was unacceptably high at the 5-FU dose level of 1,250 mg/m^2/wk on the less intensive PN401 dose schedule (schedule 1). Intensification of the PN401 dose schedule (schedule 2) permitted escalation of the dose of 5-FU to 1,950 mg/m^2/wk. However, the rate of dose-limiting hematologic toxicities was unacceptable at 5-FU doses greater than 1,250 mg/m^2/wk.

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Table 1. Patient Characteristics

Characteristic	No. of Patients (n = 23)
No. of assessable patients	22
Sex	
Male	15
Female	8
ECOG performance status	
0	13
1	10
Previous treatment	
Chemotherapy	15
Chemotherapy and radiotherapy	6
None	2
No. of prior regimens	
0	2
1	8
2	7
>2	5
Tumor type	
Colorectal	12
Gastric	2
Pancreatic	2
Prostatic	2
Renal	2
Cholecystic	1
Sarcoma	1
Vaginal	1

NOTE. Median age, 62 years; range, 39 to 81 years.
Abbreviation: ECOG, Eastern Cooperative Oncology Group.

Hematologic Toxicity

Myelosuppression, principally neutropenia, was the most common toxicity of the combination of 5-FU and PN401. Listed in Table 3 are the median and ranges of ANCs and platelet counts, as well as the rates of pertinent hematologic toxicities, as a function of dose levels of PN401 and 5-FU. The ANC nadir was typically observed on day 21 after the third weekly dose of 5-FU, although the nadir values were recorded on day 28 in three cases. However, maximal effects on platelet counts were noted on day 28, 2 weeks after the third weekly dose of 5-FU. Six patients required 1-week treatment delays during their second courses of treatment, and one patient required a 2-week delay because of unresolved neutropenia. Thrombocytopenia also contributed to treatment delays in two of these patients.

There were no episodes of DLT in patients treated at the initial dose level in which 5-FU 1,000 mg/m^2/wk was administered with PN401 (schedule 1). At the second dose level (5-FU 1,250 mg/m^2/wk), however, two of six new patients experienced DLT during course 1. These events included grade 3 diarrhea, in one patient, and grade 4 neutropenia complicated by sepsis and death, in the other patient, a 67-year-old heavily pretreated patient who had advanced pancreatic carcinoma, an Eastern Cooperative Oncology Group performance status of 1, and a normal DPD level. Another heavily pretreated individual developed grade 4 neutropenia associated with fever during courses 2 and 3. With the occurrence of these toxicities at the second dose level of 5-FU on schedule 1, the PN401 dose schedule was intensified (schedule 2).

On schedule 2, 5-FU was better tolerated and none of the three new patients treated with 5-FU 1,250 mg/m^2/wk experienced DLT. Hematologic toxicity was moderate and consisted of grade 2 neutropenia (in two courses), grade 3 neutropenia (in one course), and grade 2 thrombocytopenia (in one course). Therefore, the doses of 5-FU were successively escalated with PN401 (schedule 2) to 1,550 mg/m^2/wk and 1,950 mg/m^2/wk, which resulted in progressively lower ANC and platelet count nadirs. At the dose level of 5-FU 1,950 mg/m^2/wk, on PN401 dose schedule 2, median ANC and platelet count nadirs were 205/μL and 38/μL, respectively. In addition, both minimally pretreated patients who received 5-FU 1,950 mg/m^2/wk and PN401 (schedule 2) experienced prolonged (> 5 days) grade 4 neutropenia and grade 3 thrombocytopenia. Therefore, additional patients were treated at the next-lower dose level,

Table 2. Dose Escalation

5-FU Dose (mg/m^2/wk)	PN401 Schedule*	No. of Assessable Patients: New	No. of Assessable Patients: Previously at Higher Dose	No. of Assessable Patients: Total	No. of Courses	New Patients With DLT/All New Patients†
1,000	1	3	0	3	5	0/3
1,250	1	6	0	6	17	2/6
1,000	2	0	2	2	3	0
1,250	2	5	3	8	12	0/5
1,550	2	6	1	7	11	2/6
1,950	2	2	0	2	2	2/2

*Schedule 1: PN401 6 g every 8 hours for eight doses, starting 8 hours after 5-FU administration; schedule 2: PN401 6 g every 2 hours for three doses followed by 6 g every 6 hours for 15 doses, starting 8 hours after 5-FU administration.
†First course only.

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Table 3. Hematologic Toxicity of 5-FU and PN401

5-FU Dose (mg/m²/wk)/ PN401 Schedule*	No. of Patients	ANC Nadir (/μL)		No. of Patients With Neutropenia (no. of courses)				Platelet Count Nadir (× 10³/μL)		No. of Patients With Thrombocytopenia (No. of courses)	
		Median	Range	Grade 3	Grade 4	Grade 4 >5 Days	Grade 4 With Fever	Median	Range	Grade 3	Grade 4
1,000/1	3 (5)	3,860	1,540-6,300	0	0	0	0	285	222-414	0	0
1,250/1	7 (17)	2,220	240-9,350	0	2 (3)	0	2 (3)	240	137-671	0	0
1,000/2	2 (3)	2,500	1,112-3,030	0	0	0	0	103	95-113	0	0
1,250/2	8 (12)	1,440	700-4,200	1 (1)	0	0	0	138	60-1,640	0	0
1,550/2	7 (11)	1,750	300-4,400	1 (1)	0	2 (2)	0	108	33-202	1 (1)	0
1,950/2	2 (3)	205	110-300	0	0	2 (2)	0	38	31-44	2 (2)	0

NOTE. Values in parentheses are numbers of courses.

*Schedule 1: PN401 6 g every 8 hours for eight doses, starting 8 hours after 5-FU administration; schedule 2: PN401 6 g every 2 hours for three doses followed by 6 g every 6 hours for 15 doses, starting 8 hours after 5-FU administration.

5-FU 1,550 mg/m²/wk, along with PN401 (schedule 2), and two of three new patients experienced DLT. In both subjects who developed DLT, including a heavily pretreated patient and a previously untreated patient, ANC nadirs decreased to less than 500/μL for more than 5 days. The heavily pretreated individual also developed grade 3 thrombocytopenia. On the basis of these results, two additional new patients were treated with 5-FU 1,250 mg/m²/wk on PN401 dose schedule 2. Overall, none of five new patients, of whom three were heavily pretreated, developed DLT at this dose level.

Nonhematologic Toxicity

The rates of the principal nonhematologic toxicities of 5-FU and PN401 are listed in Table 4. Overall, nonhematologic effects were not related to the dose of 5-FU. Only one patient, a 67-year-old heavily pretreated man with metastatic colorectal carcinoma, experienced dose-limiting nonhematologic toxicity. The patient developed grade 3 diarrhea on day 15 of his first course of 5-FU 1,250 mg/m²/wk and PN401 (schedule 1). The diarrhea persisted for 5 days and was associated with dehydration and severe fatigue. In addition, 11 patients complained of grade 1 or 2 diarrhea in a total of 14 courses, which spanned four of five dose levels and both PN401 schedules. Grade 1 or 2 mucositis was experienced by six patients during seven courses and was not dose related. The onset of mucositis was generally late, occurring in weeks 3 to 4, and resolved rapidly, with only one patient at the first dose level requiring a dose reduction for this reason. Mild to moderate (grade 1 to 2) isolated elevations in serum total bilirubin level were experienced by eight patients during eight courses at four of the five dose levels. In one patient treated at the second dose level (5-FU 1,250 mg/m²/wk and PN401 dose schedule 2), the elevation of bilirubin level occurred in the context of fatal septic shock, and this event was not thought to be due to study medication. Although four of the seven subjects also had metastatic disease to the liver, progressive disease was not documented in any of these individuals. Hyperbilirubinemia was typically noted on day 21 after the third weekly dose of 5-FU and resolved completely before the next scheduled course of treatment. Grade 1 nausea and/or

Table 4. Nonhematologic Toxicities of 5-FU and PN401

5-FU Dose (mg/m²/wk)/ PN401 Schedule*	No. of Patients	No. of Patients With Toxicity									
		Mucositis		Diarrhea		Hyperbilirubinemia		Fatigue		Nausea and/or Vomiting	
		Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 2	Grade 3-4	Grade 1-2	Grade 3	Grade 1-2	Grade 3-4
1,000/1	3 (5)	3 (3)	0	0	0	0	0	2 (2)	0	1 (1)	0
1,250/1	7 (17)	0	0	2 (3)	1 (1)	2 (2)	0	1 (2)	2 (2)	4 (5)	0
1,000/2	2 (3)	0	0	0	0	0	0	0	0	0	0
1,250/2	8 (12)	2 (3)	0	3 (4)	0	3 (3)	0	3 (5)	1 (1)	1 (1)	0
1,550/2	7 (11)	0	0	4 (5)	0	2 (2)	0	2 (2)	0	2 (2)	0
1,950/2	2 (3)	1 (1)	0	1 (2)	0	1 (1)	0	0	0	1 (1)	0

NOTE. Values in parentheses are numbers of courses.

Schedule 1: PN401 6 g every 8 hours for eight doses, starting 8 hours after 5-FU administration; schedule 2: PN401 6 g every 2 hours for three doses followed by 6 g every 6 hours for 15 doses, starting 8 hours after 5-FU administration.

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Table 5. Pharmacokinetic Parameters of 5-FU in Patients Treated With 5-FU and PN401 (schedule 2)*

5-FU Dose (mg/m²/wk)	No. of Courses	AUC_{0-t} (μmol/L/h)	$AUC_{0-\infty}$ (μmol/L/h)	C_{max} (μmol/L)	V_d (L/m²)	Cl (L/h/m²)	k_e (h^{-1})
1,250	4	295 (45)	298 (44)	375 (81)	12.6 (2.23)	34 (3.94)	2.84 (0.28)
1,550	6	534. (48)	560 (64)	567 (40)	11.1 (0.67)	22.5 (2.23)	2.06 (0.22)
1,950	2	917. (65)	962 (23)	842 (7)	10.0 (1.5)	15.6 (0.38)	1.59 (0.2)

NOTE. Values are expressed as mean (standard deviation).
Abbreviation: AUC_{0-t}, area under the curve from time 0 to time of last detectable sample.
*PN401 6 g every 2 hours for three doses followed by 6 g every 6 hours for 15 doses, starting 8 hours after 5-FU administration.

vomiting was observed during 10 courses involving nine patients at all dose levels. Mild to moderate (grade 1 to 2) fatigue was reported in 11 courses (eight patients), whereas three patients experienced severe (grade 3) fatigue. The first episode occurred during the first course of treatment with 5-FU 1,250 mg/m²/wk and PN401 (schedule 1), concomitant with grade 3 diarrhea, and this event was considered a DLT. The two other individuals developed grade 3 fatigue during the first and third courses in the context of progressive disease.

Pharmacologic Studies

Thirteen patients treated with 5-FU and PN401 (schedule 2) underwent complete plasma sampling for pharmacokinetic studies of 5-FU and uridine. Technical difficulties with the assay precluded use of plasma concentration data from one subject. Plasma 5-FU concentrations peaked at the end of infusion and were less than 5 μmol/L at the last sampling time, 3 hours after infusion. The relationship between C_{max} and dose was linear ($r^2 = .71$, $P = .005$), with C_{max} values increasing from 375 ± 81 to 842 ± 7 μmol/L as the dose of 5-FU increased from 1,000 to 1,950 mg/m²/wk. Table 5 lists pertinent 5-FU pharmacokinetic parameters derived using noncompartmental methods. The pharmacokinetics appeared to be dose dependent; 5-FU Cl decreased from 34 ± 3.94 L/h/m² at the 1,250 mg/m²/wk dose level to 15.6 ± 0.38 L/h/m² at the 1,950 mg/m²/wk dose level ($P = .006$), and 5-FU $AUC_{0-\infty}$ increased disproportionally from 298 ± 44 μmol/L/h at the 1,250 mg/m²/wk dose level to 962 ± 23 μmol/L/h at the 1,950 mg/m²/wk dose level.

Plasma uridine concentrations increased with each successive 2-hour dose of PN401 to a mean C_{max} of 259.33 μmol/L 2 hours after the third dose of PN401. Thereafter, uridine concentrations were sustained above 100 μmol/L for more than 6 hours, at which time a fourth dose was administered (Fig 2). There was substantial interindividual

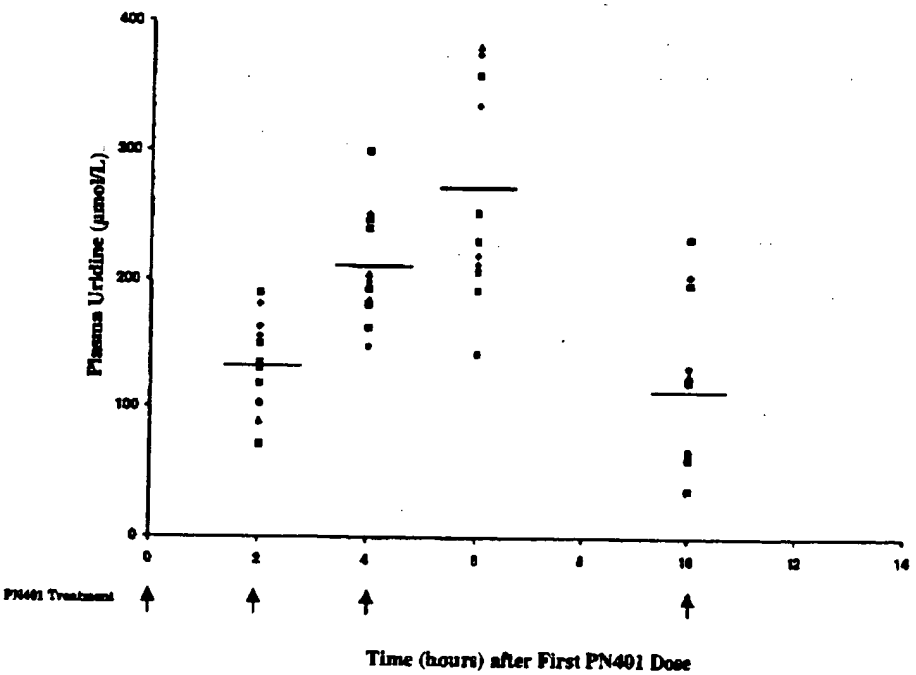

Fig 2. Plasma uridine concentrations in patients treated with 5-FU at doses of 1,250 (◆), 1,550 (■), and 1,950 mg/m² (▲) and PN401 (schedule 2).

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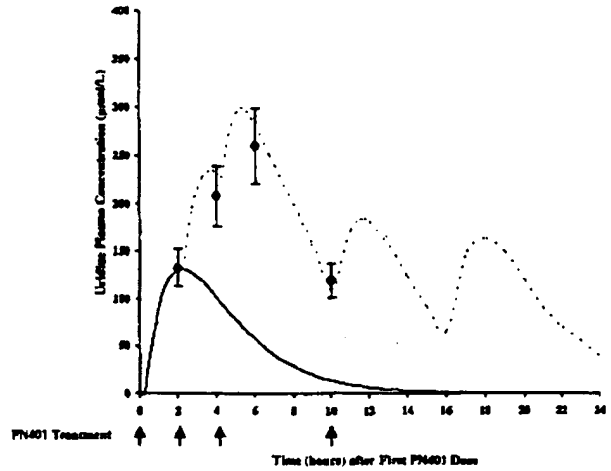

Fig 3. Mean (± SD) plasma uridine concentrations (●) after the first three doses of PN401 (schedule 2) as a function of the time after PN401 administration. The dashed line represents the fit of concentration data, applying the principle of superposition based on pharmacokinetic data derived from healthy volunteers, each treated with a single 6-g dose of PN401.

variability in plasma uridine concentrations in the 5-FU dose range of 1,250 to 1,950 mg/m²/wk (Fig 2), and no relationship between plasma uridine concentration and 5-FU dose was apparent. The plasma uridine concentrations after treatment with high doses of PN401 in this study were satisfactorily predicted by applying the principle of superposition to the pharmacokinetic data from healthy volunteers who were each treated with a single 6-g dose of PN401, as illustrated in Fig 3.

Pharmacodynamic relationships between 5-FU C_{max} and AUC and principal toxicities of 5-FU were evaluated. With regard to the effects of 5-FU and PN401 on neutrophils and platelets, interindividual variability in the percentage decrements in ANCs and platelet count was marked, and these relationships could be described with neither linear nor nonlinear models. In addition, the propensity to develop DLT was not related to peak plasma 5-FU concentrations, as demonstrated by similar C_{max} values in patients who did and those who did not experience DLT (506 ± 180 *v* 682 ± 189 μmol/L; $P = .2$ [Mann-Whitney *U* test]). In contrast, the mean $AUC_{0-\infty}$ among patients who developed DLT (703 ± 300 μmol/L) was higher, albeit not significantly, than that among patients who did not (458 ± 210 μmol/L) ($P = .1$ [Mann-Whitney *U* test]). Similarly, patients who did and those who did not develop DLT had similar maximum plasma uridine concentrations (285 ± 73 and 267 ± 74 μmol/L, respectively; $P = .4$ [Mann-Whitney *U* test]) and mean plasma uridine concentrations (186 ± 33 and 178 ± 38 μmol/L, respectively; $P = .99$ [Mann-Whitney *U* test]).

DISCUSSION

PN401, an oral prodrug of uridine, is being developed to reduce the incidence and severity of the toxicities associated with administration of 5-FU and thereby allow 5-FU dose escalation. Although 5-FU is administered on a broad range of dose schedules and no single dose-schedule has ever emerged as clearly superior, a rapid IV infusion, weekly schedule has demonstrated efficacy in several disease settings and is one of the most commonly used schedules.[1-3] By protecting hematopoietic progenitors and the gastrointestinal mucosa, PN401 may reduce the incidence and severity of unpredictable toxicities in patients receiving 5-FU and confer tolerance of high doses, resulting in greater drug exposure compared with conventional dose schedules.[13] Preclinical and prior clinical studies have demonstrated that uridine protects against the nonhematologic and hematologic toxicities of 5-FU, presumably by competing with FUTP for incorporation into the RNA of normal tissues.[4-12] In preclinical studies, the therapeutic index of 5-FU has been shown to be improved; animals treated with both 5-FU and PN401 tolerate much higher doses of 5-FU, which induce less toxicity and a greater degree of tumor regression compared with 5-FU alone.[4] However, uridine is inherently toxic and its administration is cumbersome.[9-12] IV administration requires the use of a central venous catheter to avoid phlebitis, whereas oral uridine is poorly and erratically absorbed, requiring the administration of high doses, which often result in severe diarrhea.[9-12] PN401 is an acetylated prodrug of uridine that is more lipophilic, resulting in enhanced transport across the gastrointestinal mucosa.

Whether PN401 or any other biochemical modulator will augment the therapeutic indices of the fluoropyrimidines will depend on the relative modulating effects of these agents in malignant tumors and normal tissues.[3] Preclinical studies in animal tumors have demonstrated that PN401 enhances the therapeutic index of 5-FU, provided that the agent is administered on dose schedules that result in plasma uridine concentrations of approximately 50 μmol/L, a level that is substantially higher than basal concentrations in humans (3 to 6 μmol/L).[6,13] In an early phase I study of PN401 and 5-FU, PN401, given in 6-g doses every 6 hours for a total of 10 doses beginning 24 hours after treatment with 5-FU, enabled safe administration of 5-FU in the form of rapid IV infusion at doses as high as 800 mg/m²/wk for 6 weeks, which was the recommended dose for phase II studies.[13] Plasma uridine concentrations exceeded 50 μmol/L during PN401 treatment in that study.

In the current study, we sought to optimize the biochemistry-modulating potential of PN401 by introducing two

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principal modifications in the PN401 dose schedule that was investigated in the early phase I study.[13] First, on the basis of the results of preclinical studies indicating that high doses of 5-FU are better tolerated and more efficacious when the interval between 5-FU and PN401 administration is shorter than 24 hours, the current study was designed so that treatment with PN401 commenced 8 hours after 5-FU administration. The second modification was the use of an 8-hour interval between doses of PN401 (schedule 1); in the earlier phase I study, plasma uridine concentrations exceeded 50 μmol/L for more than 6 hours after a 6-g dose. In the current study, the interval was subsequently reduced to 2 hours for the first three doses and 6 hours thereafter (schedule 2) to enhance the protective effects of PN401 after DLT consistently occurred in patients treated with 5-FU and on PN401 dose schedule 1.[13]

The results of this study demonstrate that PN401 protects against the principal toxic effects of 5-FU, confirming the results of the earlier phase I study.[13] In contrast to uridine, PN401 was well tolerated. In fact, the qualitative and temporal natures of the toxicities of the 5-FU–PN401 regimens indicate that PN401, itself, does not induce clinically significant toxicity. The toxicities of the 5-FU–PN401 regimens evaluated in the present study were qualitatively similar to those associated with 5-FU alone on a weekly schedule, with myelosuppression predominating.[1,3] The principal DLT was severe (grade 4) neutropenia that was protracted (> 5 days). Severe neutropenia consistently occurred in patients treated with 5-FU doses exceeding 1,000 mg/m^2/wk and on PN401 dose schedule 1 and in patients treated with 5-FU doses exceeding 1,250 mg/m^2/wk and on PN401 dose schedule 2. Severe nonhematologic effects were uncommon, even when relatively high doses of 5-FU were administered weekly for 6 weeks. Overall, the safe administration of 5-FU on a weekly schedule in the dosing range of 1,000 to 1,250 mg/m^2/wk, which is at least two-fold higher than the MTD of 5-FU without biochemical modulation, substantiates the potent modulating capabilities of PN401.[1-3,13] However, considering the relatively low number of patients treated at the MTD of 5-FU and PN401 on this study and the unpredictable and sometimes overwhelming toxicities of 5-FU in the individual patients, further studies are required to elucidate fully the safety and tolerability of this combination.

The potential of PN401 to protect against the toxicities of 5-FU, as well as the dose- and schedule-dependent nature of these effects, was further demonstrated as the PN401 dose schedule was intensified. With the more intensive PN401 dose schedule (schedule 2), plasma uridine concentrations progressively increased after each successive dose of PN401 given at 2-hour intervals, and plasma uridine concentrations were sustained above 100 μmol/L for most of the PN401 treatment period in most patients. This more intensive schedule permitted 5-FU doses to be increased by an additional 25% over the MTD established for 5-FU and PN401 on schedule 1. In addition, the highest safest dose of 5-FU achieved in the current study with PN401 rescue initiated 8 hours after 5-FU was 56% higher than the highest safest 5-FU dose achieved in the earlier study in which there was a 24-hour treatment interval.[13] Although the pharmacokinetics of 5-FU were not assessed in patients treated with 5-FU and PN401 on schedule 1, the nonlinear pharmacokinetics of 5-FU, as demonstrated in patients receiving 5-FU and PN401 on schedule 2 and in patients treated with 5-FU without biochemical modulation, indicate that the two-fold increase in the MTD of 5-FU due to PN401 itself, and the additional 25% increase afforded by the more intensive PN401 dose schedule, resulted in a disproportionally greater increase in 5-FU exposure. At the recommended phase II dosage of 5-FU, 1,250 mg/m^2/wk and the intensified PN401 dose schedule, 5-FU AUCs were approximately five-fold higher than those achieved without biochemical modulation.[16-21] Although the results of preclinical studies suggest that the inhibition of thymidylate synthase by FdUMP is saturated in the range of drug exposure achieved with conventional 5-FU dose schedules without biochemical modulation, the magnitude of 5-FU–induced cytotoxicity due to the incorporation of the 5-FU anabolite, FUTP, into RNA in vitro may not be saturated,[3] and this potentially important mechanism of cytotoxicity might not be taken full advantage of in standard dose-schedules.

Although the PN401 dose schedules used in the current study were selected to simulate closely those that were efficacious in preclinical studies in animals, still unknown is the optimal timing of PN401 or uridine treatment relative to administration of 5-FU to protect normal tissues maximally without protecting malignant tissue. It is of some concern that antitumor activity was not observed in the current study, despite the preponderance of patients with gastrointestinal malignancies. However, the disease of most of these patients had been demonstrated to be refractory to 5-FU treatment, with progressive tumor growth occurring during prior treatment. The 8-hour interval between treatment with 5-FU and PN401 in this study derived from findings of studies in murine colon 26 tumors.[13] In these studies, the MTD of 5-FU given weekly for 3 weeks was 100 mg/kg, which inhibited the growth of 60% of tumors, but there were no complete tumor regressions.[13] However, treatment with oral PN401 2 hours after 5-FU administration resulted in an MTD for 5-FU of 200 mg/kg and a high incidence of

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complete tumor regression. On the other hand, the MTD was 150 mg/kg when treatment with PN401 was initiated 24 hours after 5-FU administration; antitumor activity was superior to that observed with treatment with 5-FU alone, but complete tumor regressions were not observed.[13] These results support the use of shorter treatment intervals between 5-FU and PN401, such as the 8-hour interval used in the current study. However, the optimal timing between administration of 5-FU and that of PN401 is not known, and further evaluations are required to elucidate this seemingly important facet of 5-FU–PN401 administration.

In addition, although the use of PN401 and other uridine analogs for the sole purpose of increasing doses of 5-FU may not be appropriate, because the superiority of high doses of 5-FU (1,000 to 1250 mg/m^2/wk) over conventional doses without rescue (500 to 600 mg/m^2/wk) has not been firmly established in randomized trials, the principal utility of uridine-based rescue may be prevention or amelioration of the toxic effects of 5-FU in conventional-dose regimens, as well as conferring of tolerance to patients who cannot tolerate conventional 5-FU doses and would otherwise require dose reduction. The utility of PN401 in this regard is further supported by the results of studies indicating that lower doses of 5-FU (approximately 300 mg/m^2/wk) are inferior to conventional doses in patients with advanced colorectal cancer.[22-24]

In summary, the results of this study demonstrate that PN401 treatment consistently results in plasma uridine concentrations exceeding those capable of modulating the actions of 5-FU. It is also clear that the uridine exposure resulting from PN401 greatly protects normal tissues from the toxic effects of 5-FU, as demonstrated by the tolerance of 5-FU doses as high as 1,250 mg/m^2 on a weekly schedule, a level that is approximately two-fold higher than maximally tolerated 5-FU doses without biochemical modulation. Because of the nonlinearity of 5-FU pharmacokinetics, this modest increase in the MTD of 5-FU was associated with a five-fold increase in 5-FU exposure (ie, AUC). However, considering the relative low number of patients treated at the recommended phase II dose of 5-FU and PN401 on this schedule, further studies are needed to evaluate fully the safety and tolerability of this regimen. In addition, disease-directed randomized clinical trials must be performed to assess whether biochemical modulation with PN401 enhances the therapeutic indices of 5-FU in relevant disease settings.

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Phase I Trial of PN401, an Oral Prodrug of Uridine, to Prevent Toxicity From Fluorouracil in Patients With Advanced Cancer

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Purpose: We performed a phase I study to determine the appropriate dose of PN401, a uridine (URD) prodrug, to use as a rescue agent for fluorouracil (FU) and then to determine the maximum-tolerated dose (MTD) of FU when given with PN401.

Patients and Methods: Patients with advanced cancer received oral PN401 as either a suspension or a tablet in escalating doses. A pharmacokinetic analysis was performed to determine which dose best achieved a target value of sustained levels of URD ≥ 50 μmol/L. In the first phase of the study, all patients received a fixed dose of FU 600 mg/m² as a rapid intravenous bolus followed by 10 doses of PN401 given at 6-hour intervals. PN401 therapy commenced 24 hours after FU. After determination of the appropriate dose of PN401, a second group of patients received escalating doses of FU with a fixed dose of PN401.

Results: Thirty-eight patients with advanced cancer received PN401 and FU. Pharmacokinetic analysis indicated that either 6.6 g of PN401 as an oral suspension or 6 g given in tablet form resulted in high bioavailability of URD, with sustained plasma concentrations greater than 50 μmol/L. In the second phase of the study, FU doses were escalated from 600 to 1,000 mg/m². FU was given as a rapid intravenous bolus weekly for 6 weeks with a 2-week rest. The MTD of FU given in this fashion with PN401 rescue was 1,000 mg/m², at which level two of six patients had neutropenic fever. FU at doses of 800 mg/m² for 6 weeks was well tolerated without significant toxicity when given with PN401 rescue.

Conclusion: Oral PN401 is well tolerated and total doses of 6 g every 6 hours yield sustained levels of URD in the target range of 50 μmol/L. The MTD of FU with PN401 rescue is 1,000 mg/m² and the recommended dose for phase II trials is 800 mg/m² given weekly for 6 weeks with dose escalation. Further studies to define better the appropriate interval for PN401 rescue and the appropriate dose of FU when given with biochemical modulation, such as with leucovorin, are indicated.

J Clin Oncol 15:1511-1517.

FLUOROURACIL (FU) remains an important agent in the treatment of malignancy, both for adjuvant therapy of breast and colorectal cancers and in the palliative treatment of a variety of malignancies. Its mechanism of action has been extensively studied.[1] FU inhibits thymidylate synthetase via its anabolite fluorodeoxyuridine monophosphate, and also causes cell death via incorporation of fluorouridine into RNA. The major dose-limiting toxicities of FU are myelosuppression, mucositis, and, by damaging the gastrointestinal mucosa, diarrhea. These toxicities, which result in a low therapeutic index, may limit the clinical usefulness of FU.

One approach to improve the therapeutic index of FU is to decrease toxicity by using protective agents. Previous studies have demonstrated that, when given following FU administration, uridine (URD) can ameliorate toxicity, presumably by competing with FU anabolites before incorporation into cellular RNA of normal tissue, before the occurrence of irreversible cell damage. This rescue strategy has been extensively studied in animal models.[2-7] The therapeutic index of FU was increased allowing administration of higher doses of FU, which caused regression of tumors in the animal while protecting normal tissues. Preliminary clinical trials have indicated that FU toxicity can also be ameliorated in the human using either intermittent intravenous infusion of URD or, more recently, oral doses of the same agent.[8-11] However, the use of high-dose URD is technically cumbersome. Intravenous URD administration requires the use of a central venous catheter to avoid phlebitis. Oral URD is poorly absorbed and requires large amounts of the agent, which results in dose-limiting diarrhea. In two trials performed at Memorial Sloan-Kettering Cancer Center (MSKCC) that used oral URD, FU was given with PALA (PALA-FU) or with methotrexate and doxorubicin (FAMTX regi-

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Table 1. Patient Characteristics

Entered	38
Assessable	38
Male:female	18:20
Age, years	
Median	58
Range	37-73
Karnofsky performance status	
Median	80
Range	70-90
No. of prior chemotherapy regimens	31
Primary site	
Pancreas	22
Adenocarcinoma, unknown primary	11
Bile duct	2
Appendix	1
Gastric	1
Sarcoma	1

men).[11,12] There was a marked reduction in the incidence of mucositis, as well as a substantial decrease in myelosuppression. These trials also suggested that the timing of administration of URD was crucial; giving URD soon (2 hours) after FU appeared to also decrease the antineoplastic effect.

Prodrugs of URD that might allow for a more efficient delivery of the active compound have also been studied. PN401 is an acetylated prodrug of URD that is more lipophilic and thus allows increased transport across the gastrointestinal mucosa into the systemic circulation. In preclinical studies, oral PN401 resulted in eightfold higher systemic URD concentrations than did an equimolar oral dose of URD itself (D. Martin, unpublished observations). PN401 when given to mice after FU markedly decreased the myelosuppression of FU; the therapeutic index in tumor-bearing mice was improved (D. Martin, unpublished observations). These preclinical and clinical studies suggested that plasma URD levels ≥ 50 μmol/L may be necessary to protect against FU toxicity; in humans, the normal plasma URD concentration is 3 to 6 μmol/L.

We designed a phase I clinical trial, first to determine the appropriate dose of PN401 to use with a fixed dose of FU and, second, to determine the MTD of FU when given with PN401 rescue.

PATIENTS AND METHODS

PN401 (Pro-Neuron, Inc, Rockville, MD) was studied in two preparations. Initially, the drug was supplied as a powder, which was reconstituted in 45 mL of cherry syrup. Each vial contained 3.3 g of PN401 in powder form. Cohorts of three to six patients received PN401 at doses of 3.3, 6.6, or 9.9 g. A fixed dose of FU 600 mg/m^2 was given as a rapid intravenous bolus injection. Each cycle of FU was given weekly for 6 weeks followed by a 2-week rest period. PN401 rescue was started 24 hours following each FU treatment. One dose of PN401 (a total of 45, 90, or 135 mL of solution) was given every 6 hours for 10 doses. A pharmacokinetic analysis was performed after the first and ninth doses of PN401. After the first phase of the study was complete, ie, the appropriate dose of PN401 was established on the basis of a pharmacokinetic analysis (see below), the second phase to determine the MTD of FU with PN401 rescue began.

PN401 was later provided in a more palatable tablet form. Each tablet contained 750 mg of PN401. A dose of 6 g (eight tablets) was given every 6 hours for 10 doses in place of the liquid suspension. Pharmacokinetic evaluation was repeated with the tablet form to ensure that adequate URD plasma concentrations were obtained.

Following identification of the appropriate PN401 dose, escalating doses of FU were studied as follows: three to six patients were treated at each dose level. FU 700, 800, or 1,000 mg/m^2 was given as a rapid intravenous bolus weekly for 6 weeks with a 2-week rest. The MTD was defined as the dose of FU that caused grade 3 or greater toxicity in at least two of six patients. Toxicity was evaluated using the National Cancer Institute common toxicity criteria. The appropriate level of PN401 determined before dose escalation was that dose of PN401 that resulted in a sustained URD plasma concentration ≥ 50 μmol/L.

Table 2. URD Systemic Exposure (mean ± SD) Following PN401 Suspension Dosage

PN401 (g)	Patient No.	Average C_{max} (μmol/L)	Average T_{max} (hours)	AUC_{0-6} (μmol/L·h)	Average Peak (μmol/L)	Average Trough (μmol/L)	C_{ss} (μmol/L)
Dose 1							
3.3	3	91.2 ± 31.3	20 ± 0.0	348.0 ± 76.1			
6.6	3	117.7 ± 3.2	30 ± 1.0	472.7 ± 16.5			
9.9	3	204.5 ± 3.7	20 ± 1.0	791.0 ± 65.6			
Dose 9							
3.3	4				108.4 ± 25.1	37.3 ± 13.8	70.4 ± 9.6
6.6	3				161.4 ± 12.3	50.8 ± 14.0	112.1 ± 3.3
9.9	3*				292.7 ± 89.1	53.1 ± 7.9	162.5 ± 29.6

Abbreviations: C_{max}, maximum observed plasma concentration; T_{max}, time to C_{max}; AUC_{0-6}, area under the URD plasma concentration-time curve from 0 to 6 hours (estimated using the linear trapazoidal rule between consecutive plasma concentrations); C_{ss}, average steady-state URD concentrations = AUC_{0-6}/dosing interval.

*Three subjects treated, two assessable.

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Table 3. URD Systemic Exposure (mean ± SD) Following a 6.0-g PN401 Tablet Dosage Given Every 6 Hours to Seven Patients

Dose 1			Dose 9 (steady-state)		
Average C_{max} (μmol/L)	Average T_{max} (hours)	AUC_{0-6} (μmol/L·h)	Average Peak (μmol/L)	Average Trough (μmol/L)	C_{av} (μmol/L)
167.6 ± 36.9	1.8 ± 1.0	611.3 ± 122.1	201.0 ± 50.5	67.1 ± 19.1	127.9 ± 20.4

Pharmacokinetic Methods

Plasma URD concentration data were analyzed using noncompartmental pharmacokinetic methods. The area under the plasma URD concentration-time curve (AUC), a measure of extent of absorption, was estimated by the linear trapezoidal rule. Due to a limited number of time points during the absorption phase, absorption kinetics were not evaluated. The elimination rate constant (k_e) was estimated using linear least-squares fitting of the logarithm of the concentrations over the terminal phase of elimination versus time after dose 1. The maximum-observed plasma concentration (C_{max}) and the time to peak concentration (T_{max}) were obtained directly from the data without interpolation.

The prediction of plasma concentrations of URD following multiple dosing of PN401 used the principle of superposition, a method that makes no assumptions regarding a pharmacokinetic model or absorption kinetics. This method assumes that each dose of PN401 behaves independently of every other dose, that absorption, conversion to URD, and systemic clearance of URD are uniform for all dosing intervals, and that linear pharmacokinetics apply. This method also requires complete characterization of the URD plasma concentration-time profile after the initial dose. To estimate the concentration of URD after a 6-hour time point, an average k_e of 0.347 h^{-1} was used, which corresponds to an average plasma half-life of 2 hours. For prediction of plasma concentrations-time profiles for dose 2 through dose 9, the plasma concentration-time profile for dose 1 was overlaid on itself for each subsequent dosing interval and concentrations at each time were added across doses. The resultant profiles displayed in the figures are plotted with the observed plasma concentrations measured after dose 1 and dose 9 for comparative purposes.

Sampling for pharmacokinetic evaluation was performed after the first and ninth doses of PN401. Blood samples were drawn at 0, 0.5, 1, 2, 3, and 6 hours. Blood was collected in heparinized tubes and centrifuged, and the supernatant plasma frozen at −70 to −90°C for subsequent assay. Plasma concentrations of URD and uracil were determined using a reverse-phase high performance liquid chromatography (HPLC) method (HPLC system with Spectra-physics Isochrome pump, SP8490 variable-wavelength UV detector, Hitachi AS4000 autoinjector). For the tablet dosage form, plasma concentrations of URD and uracil were calculated using inosine as an internal standard. The minimum quantifiable limit was 2 μmol/L for URD and 0.4 μmol/L for uracil. Pharmacokinetic evaluation of PN401 was performed in nine patients who received the oral suspension and in seven patients who received tablets.

Patients with advanced cancer who met the following eligibility criteria were studied. All patients had a Karnofsky performance status ≥ 60%, WBC count ≥ 4,000/μL, and platelet count ≥ 150,000/μL, and adequate hepatic and renal function (serum bilirubin level ≤ 1.5 mg/dL and creatinine concentration ≤ 1.5 mg/dL). Patients may have received prior FU therapy, but could not have malabsorption syndrome or any other condition that might interfere with intestinal absorption. Signed informed consent was obtained from all patients before study entry. Pretreatment evaluation included a complete history and physical examination, evaluation of measurable disease if present, and pretreatment laboratory studies, including a complete blood cell (CBC) count and platelet count, biochemical screening profile, carcinoembryonic antigen (CEA) determination if appropriate, and chest x-ray. Imaging studies, including abdominal computed tomographic scans, were performed if indicated. During the study, an interval history and physical examination and CBC count were performed weekly. A screening profile was performed on a monthly basis. Evaluation of antitumor effect by radiographic studies, physical examination, or blood markers (CEA) was performed every 8 weeks.

RESULTS

A total of 38 patients with advanced malignancies were treated with PN401 and FU. Patient characteristics are listed in Table 1. As can be seen, slightly more than half the patients had adenocarcinoma of the pancreas. The majority of patients had not received prior treatment.

Plasma URD concentrations following PN401

Table 2 lists the pharmacokinetic parameter estimates following administration of PN401 in suspension at three different dose levels. Three patients at each level had adequate URD concentrations to allow detailed pharmacokinetic evaluation. Increasing URD concentrations were noted as dose levels increased. The mean trough value was the lowest URD plasma concentration found in blood samples drawn at dose 9, ie, 54 hours after initiation of therapy. When 3.3 g of PN401 was given, the average trough value was 37.3 μmol/L. With 6.6 g, the average trough value was 50.8 μmol/L. When 6.65 resulted in sustained URD concentrations ≥ 50 μmol/L, and myelosuppression was seen with 3.3 g of PN401 was ameliorated with 6.6 g, we chose 6.6 g for further study (see later). Table 3 lists URD plasma concentrations following 6 g of PN401 when given in tablet form every 6 hours. A total of seven patients had adequate plasma URD concentration data to allow pharmacokinetic evaluation. As can be seen, the average C_{max} was 167 μmol/L, which is well above the 117.7-μmol/L concentrations seen with the liquid preparation at 6.6 g. The average trough value

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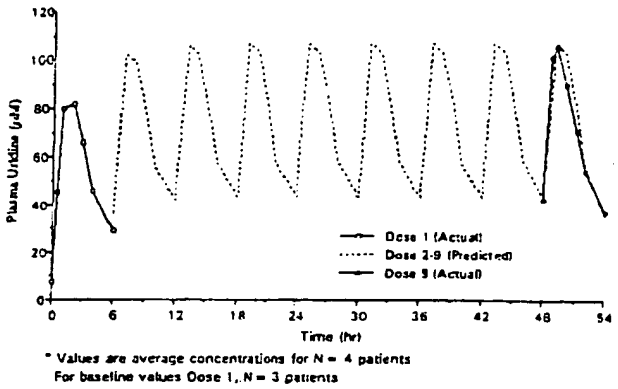
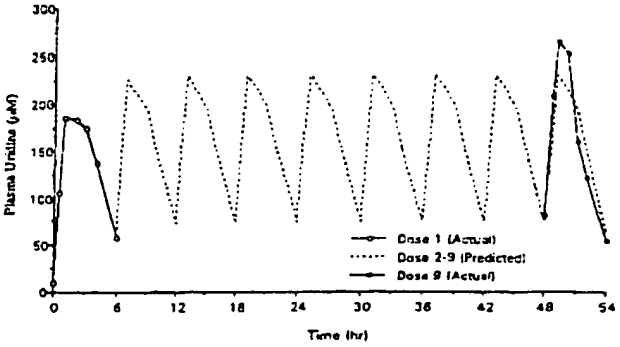


* Values are average concentrations for N = 4 patients
For baseline values Dose 1, N = 3 patients

Fig 1. Observed and predicted plasma URD concentrations following single and multiple suspension doses of PN401 (3.3 g every 6 hours).



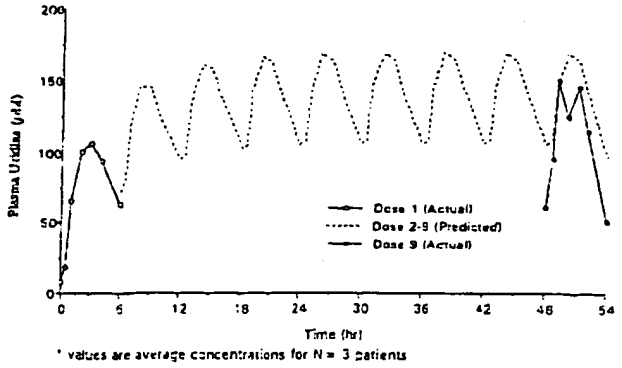
* values are average concentrations for N = 2 patients

Fig 3. Observed and predicted plasma URD concentrations following single and multiple suspension doses of PN401 (9.9 g every 6 hours).

with the tablet form was also higher (67.1 *v* 50.8 μmol/L) than that seen with the liquid preparation.

Observed and predicted plasma URD concentrations after single and multiple doses of the liquid suspension of PN401 at 3.35 and 6.6 g are shown in Figs 1 and 2. The actual measured concentrations closely approximate the predicted concentrations; sustained values greater than 50 μmol/L are obtained for 6.65. Figure 3 shows similar data for 9.95 liquid suspension. Figure 4 shows measured and predicted plasma URD concentrations when 6 g of PN401 was given in tablet form for 10 doses every 6 hours. Again, sustained levels greater than 50 μmol/L were obtained and the observed and the predicted curves are superimposable.

In summary, PN401 given at 6.6 g as a liquid suspension or as 6 g in tablet form at 6-hour intervals for 10 doses allows sustained plasma URD concentrations greater than 50 μmol/L.



* values are average concentrations for N = 3 patients

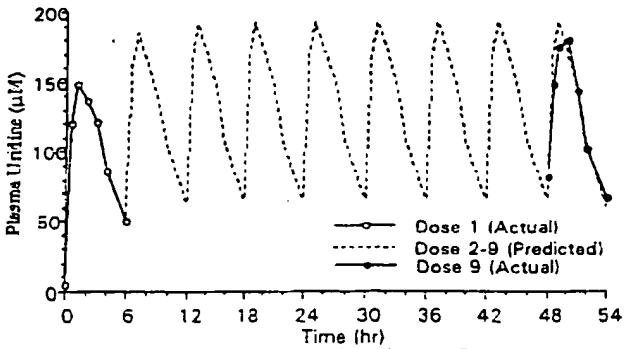
Fig 2. Observed and predicted plasma URD concentrations following single and multiple suspension doses of PN401 (6.6 g every 6 hours).

Toxicity

PN401 was well tolerated either as liquid suspension or in tablet form. The major patient complaint with the liquid preparation was taste, which, while acceptable, caused mild nausea in some patients. No diarrhea greater than grade 2 was observed with PN401 alone or with PN401 plus FU (see later). With the tablet preparation, taste was not an issue. With the liquid preparation, because each vial only contained 3.3 g of PN401, the volume of cherry syrup preparation required at 6.6 or 9.9 g was the major patient complaint.

FU Toxicity With PN401 Rescue

Table 4 lists hematologic toxicities seen by dose level expressed as median WBC count, platelet count, and hemoglobin nadir. Dose-limiting toxicity was seen at 1,000 mg/m^2. Myelosuppression resulted in admissions for neutropenic fever in two of six patients. Grade ≥ 3 neutro-



* values are average concentrations for N = 7 patients

Fig 4. Observed and predicted plasma URD concentrations following single and multiple tablet doses of PN401 (6.0 g every 6 hours).

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Table 4. Hematologic Toxicity by FU Level

FU Level* (mg/m²)	No. of Patients	Nadir Values: Median ANC (× 10³ cells/mm³)	Median Hemoglobin (g%)	Median Platelets (× 10³ cells/mm³)
600†	12	6.2	11.4	249
700	9	4.3	9.8	209
800‡	19‡	2.1	10.1	137
3.3 g PN401	7	1.1	9.9	135
6.6 g PN401	8	2.1	11	154
6 g PN401	8	3.3	10.3	194
1,000	6	0.9	8.6	160

*Five patients were treated at > 1 FU level.
†PN401 escalation phase.
‡Five of 19 patients had an ANC < 1,000 cell/μL.

penia with neutropenic fever was seen in only one of 16 patients treated with either suspension or tablet form of PN401 at 800 FU mg/m². At 800 mg/m² of FU, seven patients received PN401 3.3 g every 6 hours. The median WBC nadir was 3.4 × 10³ cells/mm³ (range, 1.4 to 6.4) and the median absolute neutrophil count (ANC) nadir was 1.1 × 10³ cells/mm³ (range, 0.1 to 4.2). We therefore treated a second cohort of patients, who received FU 800 mg/m² with 6.6 g of PN401 every 6 hours. For these six patients, the median WBC nadir was 5.0 (range, 2.3 to 8.0) and the median ANC was 2.1 (range, 1.1 to 3.6). Since 6.6 g of PN401 gave sustained URD concentrations greater than 50 μmol/L and seemed better able to ameliorate myelosuppression, we continued the study with this dose of PN401. Additional patients received 1 g/m² of FU. At this level, dose-limiting myelosuppression was seen in two of six patients. Thus, the MTD was 1,000 mg/m². Nonhematologic toxicities are listed in Table 5. Mucositis and diarrhea, the usual nonhematologic dose-limiting toxicities of FU, were rare. Similarly, no evidence of neurologic toxicity, such as cerebellar ataxia, was noted.

No chronic toxicity from administration of PN401 was noted and there was no increasing toxicity with FU seen over time. This is to say the protective effect of PN401 at a given dose level was maintained during the period that FU was given to the patient. Eleven patients were admitted for a variety of reasons during this study. As previously noted, neutropenic fever clearly related to chemotherapy was responsible for three patients being admitted to the hospital. The other admissions were felt most likely to be due to tumor-related causes, rather than to treatment.

Table 5. Nonhematologic Toxicity Greater Than Grade 3

Dose Level (mg/m²)	No. of Patients: Mucositis	Diarrhea	Nausea/Vomiting	Fatigue
600	0	0	0	1
700	0	0	0	1
800	0	0	1	0
1,000	0	0	0	0

Tumor Response

As noted earlier, the majority of patients in this study had pancreatic carcinoma. One major response was seen, which was fairly durable (12 months). No other major responses occurred among patients with pancreatic carcinoma. Many patients did not have measurable or assessable disease and therefore it was not possible to assess objective response.

DISCUSSION

FU remains an important agent in the treatment of a variety of solid tumors, particularly in the adjuvant setting. While active new agents of other classes have been identified in the treatment of both breast tumors (paclitaxel) and more recently in colorectal tumors (irinotecan and tomudex), FU remains an integral component of therapy for breast cancer in the cyclophosphamide, doxorubicin, and FU (CAF) or cyclophosphamide, methotrexate, and FU (CMF) adjuvant regimens and of colorectal cancer both in the adjuvant setting and for advanced-disease patients. From phase I and II trials performed primarily in the 1970s, the MTD of FU as a single drug is 500 to 600 mg/m²/wk for 6 weeks. Higher doses have occasionally been used. Fraile et al[13] treated five patients with one dose of 600 to 900 mg/m² of FU as a rapid intravenous injection. The study, which was primarily a pharmacokinetics analysis, noted that three of five patients developed leukopenia after a single treatment with high-dose FU.

Previous studies have demonstrated that URD can ameliorate the hematologic and nonhematologic toxicities of FU when given either intravenously or orally. Dose-escalation trials of biochemically modulated FU with oral URD have been performed at MSKCC. In these studies, a substantial increase in the delivered dose of FU in the FAMTX regimen was achieved.[12]

Similarly, Seiter et al[11] demonstrated that using oral URD, high weekly doses of PALA-modulated FU could be given (the MTD of FU was 1,100 mg/m²/wk for six doses). However, in both of these studies, the large number of URD tablets required was poorly tolerated.

In the current trial, we have demonstrated that PN401 is well tolerated and has a superior URD bioavailability

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to URD tablets. When given 24 hours following administration of FU, PN401 almost completely blocks the nonhematologic toxicities of mucositis and diarrhea. Dose escalation of up to 1,000 mg/m^2/wk could be achieved.

Because FU clearance is reduced at higher doses due to saturation of its degradative enzymes and excretory pathways, the AUC of FU at 1,000 mg/m^2 is actually threefold to fourfold higher than that of the normal FU dose of 600 mg/m^2.[14] FU cytotoxicity via RNA-directed mechanisms in vitro appears to be a function of the concentration-time product of FU exposure, which is equivalent to the AUC in vivo. The improvement in antitumor efficacy obtained by FU dose escalation followed by URD rescue has been attributed to a net increase in FU incorporation into tumor RNA.[7] Thus, lower doses of FU alone in mice and humans may be insufficient to exploit the potential antitumor efficacy of RNA-directed cytotoxicity in addition to that due to inhibition by FdUMP of thymidylate synthase, a mechanism that is saturated at relatively low doses of FU.

Several crucial questions remain unanswered. What is the optimal timing of PN401 to avoid rescue of tumor, but to allow protection of normal tissues? A 24-hour interval was chosen in this study on the basis of our data from the FAMTX URD trial.[12] In that study, we initially began URD rescue 2 hours after administration of rapid intravenous bolus FU. We noted that although there was protection from toxicity, no responses were seen using a regimen that ordinarily induces remissions in approximately 30% of patients with gastric cancer. We hypothesized that URD given 2 hours after FU might also rescue tumor, as well as normal tissue. When we increased the interval between FU administration and URD rescue to 24 hours, major objective regressions were seen in patients with measurable disease (similar to those expected from FAMTX without URD), but with substantially less toxicity. Particularly striking was the experience in a single patient who had no response after receiving FAMTX with URD rescue 2 hours after administration of FU, but who had a complete clinical remission when the interval between FU and URD was extended to 24 hours. This patient, with advanced gastric cancer, received three cycles of FAMTX therapy with URD given 2 hours after treatment with FU. There was no objective response after 2 ½ months of treatment. On her fourth cycle (2 weeks after evaluation showed stable disease), the interval between FU (modulated by methotrexate in the FAMTX regimen) and URD was increased to 24 hours. A complete clinical remission was documented by repeat computed tomographic scan and repeat endoscopy. The patient remained in complete remission for 7 months. However, the decision to treat at 24 hours was made empirically. D. Martin (unpublished observations) has extensively studied PN401 and URD rescue of FU toxicity in an animal model. In preclinical studies with advanced colon 26 murine colon carcinoma, the MTD of FU on a weekly-times-three schedule is 100 mg/kg, which results in a 60% inhibition of tumor growth and no regressions. Oral PN401 administered 2 hours after FU permits dose escalation to 200 mg/kg of FU, which resulted in a high incidence of durable complete regressions. Initiation of PN401 administration 24 hours after FU resulted in an MTD for FU of approximately 150 mg/kg, which was not sufficient to produce tumor regression, although the tumor growth inhibition was better than that produced by FU alone at 100 mg/kg. Further studies are needed in humans to determine the timing of PN401 administration relative to FU that results in an optimum balance between FU dose-intensity and retention of selectivity of rescue for normal tissues, but not tumors. It is possible that initiation of PN401 rescue greater than 2 hours but less than 24 hours after FU will allow further dose escalation of FU while preserving antineoplastic activity. In addition, the predicted steady-state plasma concentration data for multiple doses of PN401 would support extending the dosing interval to 8 hours in future clinical trials.

The duration of PN401 therapy was also decided empirically. FU has a short plasma half-life. It is possible that rescue is required for a period less than 60 hours. Future trials should explore differences in the duration of PN401 rescue.

In summary, PN401 rescues FU hematologic and nonhematologic toxicities and allows the use of substantially higher doses of FU on a weekly schedule. Phase I/II trials using FU at a starting dose of 800 mg/m^2 with PN401 6 g orally for 10 doses at 6-hour intervals beginning 24 hours after initiation of FU in FU-sensitive tumors such as colorectal cancer are appropriate. Additional studies to identify the appropriate dose of FU to use with biochemical modulation (such as leucovorin or PALA and methotrexate) are also indicated. Since modulating agents such as leucovorin almost invariably require dose attenuation of FU to prevent unacceptable toxicity, it is possible that PN401 may allow higher doses of FU to be given, while still allowing biochemical modulation to be performed.

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